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REPORT NO. 4

DEVELOPMENT OF AN ON-LINE BIOLOGICAL DETECTOR

Annual Summary Report
July 1, 1975 - June 31, 1976

K. Ehrlich, Ph.D., E. Klein, Ph.D.,
and J.K. Smith, M.S.

July 31, 1976

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
WASHINGTON, D. C. 20314

Contract Number DAMD 17-⁷⁵~~76~~-C-5075

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) We are growing mammalian cells in artificial capillary perfusion culture in an effort to develop a sensor for toxic components in recycled water intended for human consumption. Cultures of BHK or L929 cells can be established in capillary culture in one week or less by planting the units with a high seed population. Culture using either 1% or 10% calf serum-containing medium allows the cells to reach a plateau in growth. Cultures can be maintained in either a once-through perfusion mode or a recirculation perfusion mode for prolonged periods at rates slow enough to allow rapid measurement of oxygen and glucose utilization and (over)		

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Lactic acid excretion rate provided that (1) the flow rate is slow enough and (2) the toxin is in direct contact with the cells. To meet the latter condition, we have investigated a new design for perfusion where the shell containing the cells is directly perfused with growth medium.

SUMMARY

A rapid, sensitive detector of toxic components in recycled water intended for human consumption is being developed by GSRI for the U.S. Army MUST program. In our first year of effort we have investigated the feasibility of using metabolic changes of mammalian cell cultures on artificial capillary units as the detector response.

Activities directed toward this goal in the first year have included (1) growth of cells on highly permeable capillary units, (2) the optimization of the response times of cells on these units, both for detection of metabolic activity and for assessment of toxic response, and (3) the measurement of response to certain selected toxins. These activities are prerequisite to successful development of a detector prototype.

We have investigated methods to establish, rapidly, a population of metabolically stable cells sufficient for metabolic rate measurement, on the units. These methods include planting of high cell numbers per unit and the use of medium containing a low serum content. Metabolic activity of cells on artificial capillary units has been assessed by measuring the rate of glucose and oxygen utilization from, and the lactic acid excretion into, the perflusate medium of L929 and BHK cells on the units. Variation of metabolic activities with (1) time in culture, (2) flow rate, (3) medium serum concentration, and (4) toxin dosages has been followed. Different perfusion modes have also been studied. Capillary culture units have been perfused (1) by medium recirculating from a reservoir through the capillary bore, (2) by medium passing through the capillary bore and then to waste, and (3) by medium which is passed through the shell and then discarded.

Our initial findings include the following: The metabolic activity of the cells is dependent on the perfusion flow rate. Cultures of BHK and L929 cells can be maintained for extended periods in medium containing low serum (1%) using either once-through or recycled flow. To date, the best mode of operation for measurement of rapid toxic response is to pass medium containing toxin through the shell of the artificial capillary unit. When this mode was used, the change in cellular metabolic activity upon introduction of KCN,

o-toluidine, and iodoacetate into the medium was sensed almost immediately after introduction of the toxin within the capillary unit shell. In some cases, the change in metabolic activity could be entirely reversed by subsequent perfusion of fresh medium (not containing toxin) through the shell. The best and most sensitive means of assaying cellular metabolic activity is by oxygen utilization.

The results of our first year of investigations indicate that stable populations of either 1929 or BHK cells can be maintained on the capillary units for extended periods, that response to certain toxins can be detected within 5-15 min after introduction of toxin, and that cell recovery after brief exposure to toxins is possible.

1. INTRODUCTION

In portable field hospitals under development in the U.S. Army MUST program, it is intended that the water supply will come only from wastewater purified by a multiple treatment process including reverse osmosis and ozonation. The purification will require that the water supply contain no elements that can be immediately or subsequently toxic to humans. In order to insure that the toxin level in the water is low enough for safe human consumption, a biological detector of toxicity is being developed by Gulf South Research Institute. Such a detector must be capable of rapid response and sufficient sensitivity. We plan to use a detector in which mammalian cells are maintained on artificial capillary tissue culture units¹ by a perfusate composed of growth medium mixed with the water stream to be tested. A deviation in the cellular metabolic activity below or above a threshold (to be determined) would indicate a toxic response. Should a toxic response be obtained, the water stream would be diverted from the potable water reservoir. The response of animal cells in culture to toxins incorporated in the growth medium has already been shown to be a valid method for testing the relative toxicity of a chemical².

1.1 FIRST YEAR OBJECTIVES

The first-year goals included selection of tissue culture cell types suitable for monitoring metabolic activities of the cells on the artificial capillary units, establishment of cell populations on the units, and measurement of the response of these cells to selected toxins. We propose to devise a flow-through detector capable of a rapid response to toxins, and capable of good instrumental control.

1.2 BACKGROUND

1.2.1 Perfusion Cell Culture

It has been widely recognized that culture of cells as mono- or multi-layers in flasks using only periodic medium changes to supply nutrients is suboptimal. There are several reasons for suboptimal culture under such conditions: the growth environment varies with time after feeding due to nutrient depletion by the cells (alternate feasting and fasting), a matrix for three-dimensional growth cannot be built up, the medium must be "reconditioned" after each feeding period, and cell growth to tissue-like densities cannot be achieved.

Because these limitations in conventional flask culture have been recognized, several perfusion systems for cell culture have been designed¹. Some of these early methods borrowed techniques from microbiological research, namely the chemostat². Such techniques were designed for continuous growth of cells in suspension. Perfusion systems for monolayer culture were initially designed primarily for microscopic work^{3,4,5,6}. A perfusion system for culture flasks has been described by Kruse et al.⁷. This system allows cells to grow to tissue-like densities (up to 18 cell layers thick) in culture flasks. Kruse's studies with thirteen different cell types showed that these cell types are able to form multilayers under perfusion conditions. Kruse also showed with WI38 cells that a nonmitotic cell population could be maintained for almost one month by lowering the medium serum content from 10 to 0.1%⁸.

Although it is generally difficult to correlate glucose uptake, cell numbers and rates of cell proliferation in static flask cultures, Kruse and Miedema were able to make such correlations in perfusion flask cultures⁹. As the cell population went from a preconfluent density to postconfluent density, the change in the rate of proliferation was accompanied by a proportionate change in the rate of glucose uptake. Rates of glucose uptake correlated well with proliferation rates for a number of fibroblast cell lines. The average rates of glucose uptake and lactic acid production in postconfluent cultures of WI38 and transformed WI38 cells were the same as in preconfluent

cultures. Kruse calculated that on the average for a variety of cell types in perfusion culture the glucose consumption was roughly $5 \mu\text{mol}/10^6$ cells per day ($0.208 \mu\text{mol}/\text{hr}$ per 10^6 cells) and lactic acid production was $9.5 \mu\text{mol}/10^6$ cells per day ($0.396 \mu\text{mol}/\text{hr}$ per 10^6 cells).

The perfusion systems described above all allow direct medium contact with the cells. This insures that serum proteins necessary for cell growth will be able to interact with cell surface receptors and that cellular excretion products which might interfere with cellular proliferation can be continuously removed. The only limitation to cell growth in such systems is the requirement that the bottom cell layer receive adequate nutrients for growth.

1.2.2 Perfusion Cell Culture on Artificial Capillary Units

In 1972 Knazek and coworkers described a perfusion system which used semipermeable hollow fibers in a tube-and-shell configuration to support cell growth¹⁰. Since then a number of reports describing artificial capillary systems for cell culture have appeared¹¹⁻¹⁷. Diploid cells^{12,13}, transformed cells^{10,15} and other cell types¹⁶ have been successfully maintained on such units for periods as long as 3 months. Tissue-like densities can be achieved¹⁰ and hormone production per cell can exceed that to be expected from the same cells maintained in flask cultures¹¹.

In the tissue culture mode described by Knazek, et al.^{10,11} medium from a reservoir is circulated through hollow fibers which are sealed into a shell and then back to the reservoir. The cells adhere to the outside of the fibers and are fed by nutrients from the medium, including oxygen which diffuses through the fiber wall. The deoxygenated medium passing back to the reservoir is reoxygenated by atmospheric oxygen diffusing through silastic tubing that is used to connect the various components of the perfusion system. The flow rate (maintained by a peristaltic pump) is adjusted to supply medium to the units at a rate fast enough to maintain a constant oxygen delivery to all cells in the unit. Cell growth on the units can be judged by determining cell number after trypsinization or by determining DNA content¹⁰. Alternatively, cell growth can be estimated from the rate of glucose utilization¹⁴.

With such a system, at any one time only a small amount of medium is in contact with the cells. Nutrients must pass through the fiber walls to feed the cells, but apparently this requirement represents no restriction to the growth of most types of cells, especially in units containing fibers that are permeable to molecules of molecular weight 30,000 to 100,000. In fact, such limited permeabilities could be advantageous; soluble collagen secreted by fibroblast cells might not be lost upon medium change as would occur in conventional flask cultures. Therefore, the collagen might remain to supply a matrix for further cellular support. With artificial capillary perusion cell culture, medium must be replaced periodically as nutrients in the reservoir supply become exhausted and as lactic acid builds up. Whether or not periodic change of the shell medium is necessary has not been discussed in the above studies but in recent work (at least with some cell types), such routine replacement of the shell medium does not seem to be necessary (Knazek, unpublished results).

2. CONSTRUCTION OF A TISSUE CULTURE MONITOR

The tissue culture monitor which we plan to construct will use the change in the metabolic rate of nondividing mammalian cells after exposure to toxins in the medium to indicate the biohazard associated with the water supply which is used to dilute the medium. The cells will be grown on artificial capillary units and perfused by a medium reconstituted with the water supply to be tested. Determination of the feasibility and sensitivity of this scheme for toxin monitoring required (1) study of the growth of cells on artificial capillary units, (2) construction of new artificial capillary units, (3) investigation of different methods which sense metabolic response (4) adaptation of these methods for rapid detection of metabolic rate changes, and (5) study of the performance of different perfusion systems for introduction of toxin to the cells. These aspects of the first year research program will be discussed in detail below.

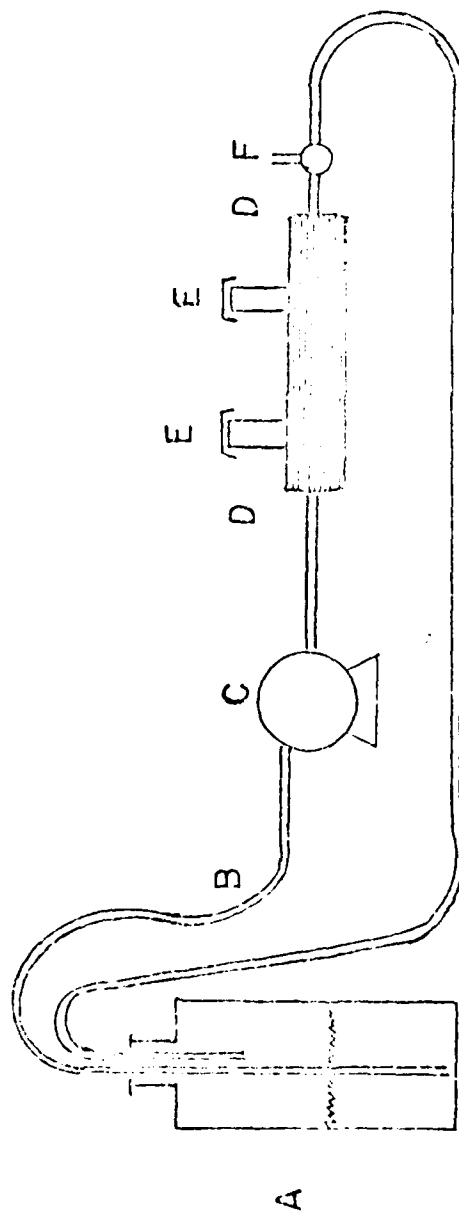
2.1 GROWTH OF CELLS ON ARTIFICIAL CAPILLARY UNITS

2.1.1 Perfusion System for Cell Growth

Initial efforts to grow cells on capillary units were based on the culture conditions reported in the literature, which use a perfusion circuit similar to that of Figure 1. In such a circuit, growth medium contained in a reservoir is circulated by a peristaltic pump to the cell culture unit and then returned to the reservoir. The medium flows through the bore of the fiber at flow rates from 0.7 to 5 ml/min. The cells adhere to the capillary surface in medium contained in a shell surrounding the fibers (cf. Fig. 1). As of July 1975, no literature suggested any dependence of culture success on flow rate or fiber type although proper oxygenation of the medium was stated to be a principal factor in the performance of the capillary unit¹¹. The MUST detector system dictates that the medium perfusing the culture unit should flow to waste rather than return to the reservoir. It is clear that, if such a system is to be feasible, the flow rate must be sufficiently slow to allow cellular metabolic activity to be monitored and to prevent large expenditures on medium.

FIGURE 1

Diagram of the Perfusion Circuit



- A - Reservoir suitable for 200 ml medium
(Cheateon square culture bottle fitted with a #3 2-hole rubber stopper)
- B - Silastic medical grade tubing (2042 x 2.125 in., 2.194 x .192 in.) total length 3 ft.
- C - Peristaltic pump (Buchler 4-channel, Brinkmann 6-channel, Technicon 8-channel, Sigmameter 1-channel)
- D - Luer fittings
- E - Septum Stoppers
- F - Luer - 3-way stopcock

Whether slow perfusion of medium would adversely affect cell growth or maintenance was unknown.

At the beginning of this first year of research, we tried to culture several different types of mammalian cells (human foreskin fibroblasts in secondary culture, 3T3 mouse fibroblasts, L929 and primary porcine kidney cells) on several different kinds of capillary units with media perfused at slow flow rates (~ 0.10 – 0.05 ml/min). Approximately 1 – 5×10^6 cells were planted in the extracapillary space in the units and the units were perfused by medium from a small reservoir. Perfusion for some units was to waste, and in others the medium was recirculated. (The recirculation mode was tried to ascertain whether or not "conditioning" of the culture medium was critical for cell growth.) In both cases after a number of weeks, very little growth was evident from glucose and lactic acid assays of the culture media. Only 4 – 5×10^6 cells could be recovered from the units by trypsinization. These results indicated that our culture conditions were possibly unsuitable for growth of a large population of cells.

Since it was possible that cells were not receiving sufficient oxygen to support a large population when slow flow rates were used we next allowed the medium to circulate at flow rates of 2 – 5 ml/min using the perfusion mode of Figure 1. In one case, 62×10^6 BHK (Baby Hamster Kidney) cells were recovered after 8 days from a GSRI cellulose acetate fiber unit initially loaded with 5×10^6 cells. In another case, 47×10^6 cells were recovered from an Amicon 3S100 unit after 15 days of growth from an initial seeding of 5×10^6 cells. Other units also showed substantial growth over a two-week period as evidenced by glucose utilization from the perfusing medium. During these periods of growth, the reservoir medium of 100 ml of Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal calf serum (FCS) was refreshed every second day.

Currently, we are using the following protocol to establish a population of cells on an artificial capillary unit. The unit is perfused by medium in a reservoir as in Figure 1. The medium is recirculated at greater than 2 ml/min until its pH becomes too acidic (pH 6.8) or the glucose concentration falls below 1 mM. The medium in the reservoir only, is then replaced. The cultures are initiated by introducing cells into the extracapillary space

in the shell by syringe with no medium flowing and with the exit and inlet tubing clamped. Sufficient medium is included to fill the fluid space in the shell. Approximately $30-100 \times 10^6$ cells are loaded. After 0 to 30 min is allowed for the cells to adhere, the peristaltic pump is started and perfusion is begun. The reservoir contains 100 ml of DMEM supplemented with 10% calf serum (CS), 20 mM Hepes, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml chlorotetracycline, 50 µg/ml mycostatin. The medium contains 0.35 g/l sodium bicarbonate and an additional 2 g/l sodium chloride is added to maintain osmolality. The medium pH is 7.3. The culture units including pump and reservoir are maintained at 37°C in a warm room. Connections between unit, pump, and reservoir are made by a total of 1 meter of silastic tubing. A three-way Luer nylon stopcock is included in the circuit to aid in sampling. The circuit is shown in Figure 1. The medium used for subsequent culturing does not contain mycostatin. After a plateau in growth is reached as determined by a plateau in the rate of glucose uptake and/or lactic acid excretion, the medium is supplemented with only 1% CS and antibiotics. After three weeks of growth in 1% CS and a total of six weeks of growth, no deleterious effect on the cells was observed due to the reduced concentration of calf serum. We have found that cells can be grown to high densities even when the cultures are initiated using medium with 1% CS. Typical growth curves for BHK and L929 cells maintained for over three weeks are shown in Figures 2 and 3, respectively. Both of these cultures were initiated using medium containing 10% CS. At the arrow in Figure 2, the same medium supplemented with only 1% CS was used. Flow rates were 8.5 ml/min for the L929 cells (Fig. 3) and 3.0 ml/min for the BHK cells. A plateau in the rate of glucose utilization and lactic acid excretion is reached on the average within one week after the units are planted with a large number of cells (30×10^6 or greater). At the termination of these studies, cells were recovered from the units by trypsinization. More than 6×10^7 L cells and 5×10^7 BHK cells were recovered.

When this protocol is used, planting success with BHK and L929 cells is essentially 100% provided that no leakage of cells through the potting at the end of the unit occurs after flow is initiated. Most of the units reached cell populations with an average lactic acid excretion rate of at least 12

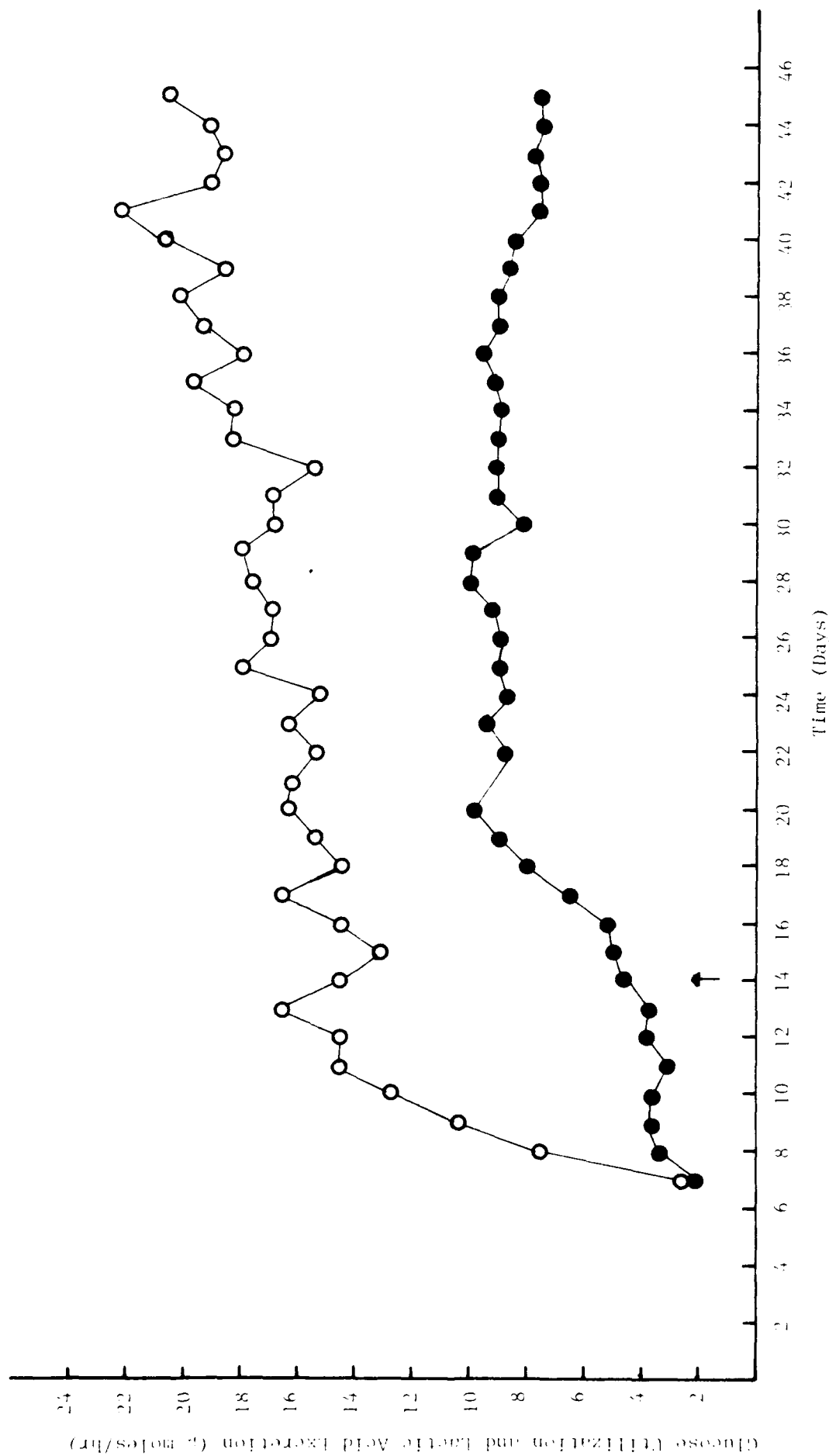


Fig. 2. Growth of BHK cells on a capillary unit.

0-0, lactic and excretion, $\mu\text{mol/hr}$; $\bullet-\bullet$, glucose utilization, $\mu\text{mol/hr}$. Perfusion was as shown in Figure 1 on a GSRI unit (#2) at 3.0 ml/min with DMEM, 10% calf serum; 20 mM Hepes, and chlorotetracycline 100 $\mu\text{g/ml}$. At day 14, the medium with only 1% calf serum was used. 100×10^6 BHK cells were planted on day 0, and 50×10^6 cells recovered on day 45. The average glucose utilization rate over days 10-45 was $7.9 \pm 1.0 \mu\text{mol/hr}$ and the lactic acid excretion rate was $17.6 \pm 1.9 \mu\text{mol/hr}$.

$\mu\text{mol/hr}$ and a glucose uptake rate of $8 \mu\text{mol/hr}$. In some cases higher rates were seen.

BHK and L929 cells were chosen for these studies to test the behavior of a cell type subject to density-dependent inhibition of growth in flask culture (BHK)¹⁸ and a cell type which shows no such growth control but which ceases proliferation when medium nutrients become depleted or when access to these nutrients is inhibited due to cell overgrowth¹⁹. We also wanted to test a diploid cell type with a finite life span (human foreskin fibroblast), but poor growth prevented these studies. Both BHK and L929 seem able to adapt readily to growth on artificial capillary units as evidenced by their initial rapid growth immediately following planting (Figs. 2 and 3). Part of this ready adaptation may be due to a beneficial stimulus provided by the perfusion system which overcomes any negative effects on growth caused by the change from a support matrix of treated polystyrene to permeable polysulfone or cellulose acetate. Since artificial capillary perfusion culture geometrically limits cell growth to a certain density, we were not sure how well a cell type subject to density-dependent inhibition of growth might grow in capillary culture. We were also concerned that L929 might reach a certain limiting density and then rapidly die back to allow new growth. In both cases the growth curve is stable after the first week (Figs. 2 and 3). This stability suggests that, regardless of whether the cells limit their growth by death and proliferation or by being locked into the G_0 phase of the cell cycle¹⁹, the average net metabolic rate (measured as oxygen and glucose utilization or lactic acid excretion at the exit stream from the culture unit remains essentially constant. Our oxygen utilization studies, described below (section 2.5.2.2), also verify that metabolic rate does not fluctuate rapidly when the flow rate is constant. This fact is amenable to the choice of oxygen utilization as the metabolic parameter to be sensed in the prototype biological detector. We will continue to use both cell lines for evaluation of the biological detector. Toxicity parameters have been well worked out for L929 by other workers^{20,21} and BHK cells have a number of differentiated functions (proteoglycan synthesis, collagen synthesis) that might affect their sensitivity to toxins. Both cell lines can be maintained with only 1% serum in the medium. If an increase in toxin sensitivity is desired, even lower

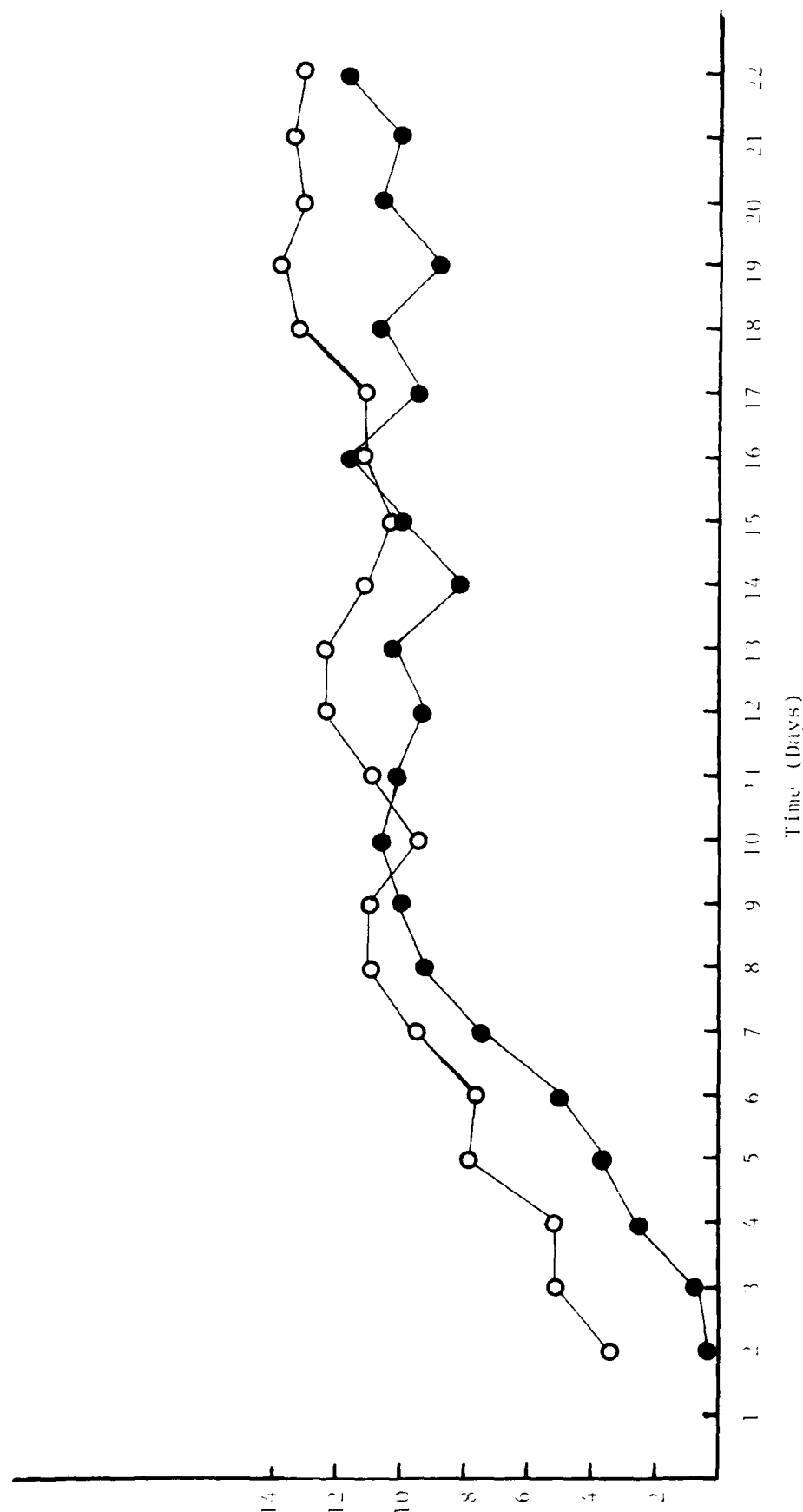


Fig. 3. Growth of 1929 on a capillary unit

0-0, lactic and excretion (μmol/hr). ●-●, glucose utilization, μmol/hr. Perfusion was as shown in Fig. 1, using an Amicon 3S100 unit at 8.5 ml/min with DMEM, 10% CS; 20mM Hepes and chlorotetracycline (100 μg/ml). 20×10^6 cells were planted on day 0; 60×10^6 cells were recovered on day 20. The average lactic acid excretion rate for days 6-20 was 12.3 ± 1.0 μmol/hr and the average glucose utilization rate over the same period was 9.9 ± 0.9 μmol/hr.

concentrations of serum will be used. Kruse showed that, under perfusion conditions, even the diploid cell line WI38 could be maintained for weeks (without cell division) in medium containing 0.1% serum¹.

2.1.2 Perfusion System for Toxicity Monitoring

The perfusion scheme shown in Figure 1 is being used to initiate cell cultures on artificial capillary units. Cultures which show no further increase in metabolic rate as indicated by a plateau of their growth curves (cf. Figs. 2 and 3, day 7 and after) are then used for toxicity studies or the once-through perfusion studies described below (section 2.5.2).

The Luer fittings on the capillary units (indicated by D in Fig. 1) enable the units to be moved from one perfusion system to another. The once-through perfusion circuits shown in Figure 4 has Luer fittings at all the capillary unit entrance and exit ports. In Figure 4a the perfusion scheme is shown for once-through flow through the fiber bore of the capillary unit, and Figure 4b shows the scheme for once-through perfusion of the capillary unit shell. In both cases the outflow medium is monitored. It may be monitored continuously as by oxygen or pH sensing or it may be sampled as with glucose and lactic acid content determinations. The nutrient content in the inlet stream to the capillary unit is determined only once in the course of an individual experiment. After it is monitored, the medium is discarded into a waste medium receiver. The flow rate is monitored periodically by measuring the rate at which the outflow medium fills a 1-cc syringe. More elaborate flow meters could also be used.

In both perfusion schemes shown in Figure 4, the flow rate is adjusted so that, after the capillary unit is connected, the outlet pO_2 is between 30 and 80 mm Hg. For some experiments flow rate was adjusted to allow detection of the glucose and lactic acid content of the medium. We are currently attempting to devise a perfusion scheme that will allow simultaneous glucose, lactic acid, and oxygen detection.

The sample to be tested for toxicity will be mixed with tissue culture medium and introduced at the 3-way stopcock (B, Fig. 4). Change to the perfusion stream containing only the uncontaminated medium can be made by simply

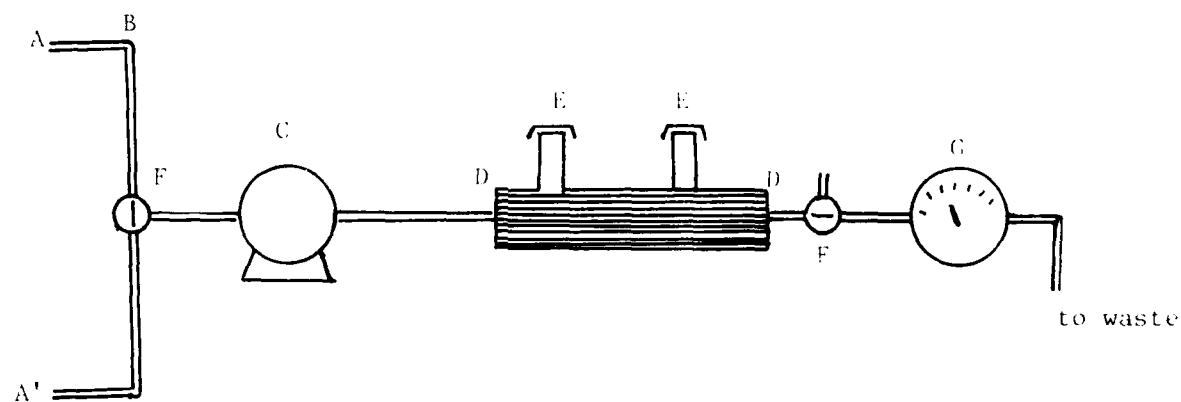


Fig. 4a. Perfusion scheme for once-through flow.

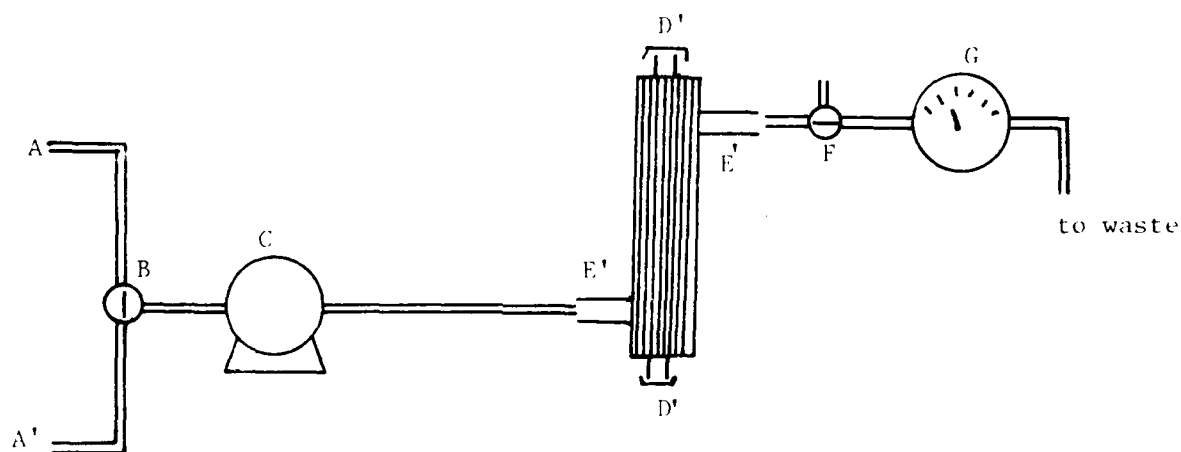


Fig. 4b. Perfusion scheme for once-through flow.

- A - reservoir for tissue culture medium
- A' - reservoir for tissue culture medium plus toxin
- B - silastic tubing
- C - peristaltic pump
- D - male Luer connectors
- E - female Luer to closed end
- E' - female Luer connectors
- F - Luer - 3-way stopcock
- G - oxygen meter

turning the stopcock. With these schemes, there is no need for the interruption of flow between exposure to toxin and exposure to recovery medium. Results using such perfusion schemes will be described in detail below (section 2.5).

2.2 METHODS TO DETECT METABOLIC ACTIVITY

We have been monitoring cellular metabolic activity by assessing the rate of glucose and oxygen utilization and lactic acid excretion. Medium glucose and lactic acid content is determined by enzymatic methods using glucose oxidase²² and lactic dehydrogenase²³. Oxygen content is determined by oxygen-specific electrode.

For determination of glucose we are using a test kit supplied by Sigma chemical (Kit #510). This kit uses the enzymes, glucose oxidase (Aspergillus niger) and peroxidase (horseradish). In the presence of glucose oxidase, D-glucose is converted to gluconate and hydrogen peroxide. The hydrogen peroxide released (1 mol for each mol of glucose) is used to oxidize the oxygen-donor, o-dianisidine dihydrochloride, to a brown product whose absorption is measured at 450 nm. A 1 mg/ml solution of glucose in water is used as the standard. The amount of glucose/ml is calculated by dividing the optical density at 450 nm for the test sample by the optical density at 450 nm for the glucose standard. The coefficient of variation for this test performed on a 1 mg/ml glucose solution assayed over 13 days was 4.9%.

Lactic acid analysis was performed using Sigma kit 826-UV. The procedure designed for blood lactic acid measurement was modified as follows: 2 ml of glycine buffer (0.4 M hydrazine; 0.5 M glycine; pH 9.0), 4 ml of water and 0.1 ml of lactic dehydrogenase (~5 mg protein/ml) and 10 mg NAD (nicotinamide-adenine dinucleotide) are combined. To 0.9 ml of this solution is added 0.05 ml of sample. The sample is prepared by adding 2 volumes of cold 8% HClO₄ to 1 volume of the medium to be tested and the cold mixture is centrifuged at ~500 rpm for 10 min. After this centrifugation, the sample is the supernatant. The reaction mixture is allowed to incubate at 37°C for 30 min. During this time the lactic dehydrogenase converts one molecule of L-(+)-lactate to one molecule of pyruvate (which is trapped as the hydrazone).

Simultaneously, one mol of NAD^+ is converted to one molecule of NADH. The absorption of NADH at 340 nm is measured. Lactic acid concentration is calculated from the millimolar extinction coefficient for NADH at 340 nm, the optical density, and the reaction volume by the equation:

$$\text{lactic acid (mMole/l)} = \frac{(A_{340})(0.95 \text{ ml})}{6.22 (0.01667)(1)}$$

where A_{340} = final maximum absorbance at 340 nm

0.95 = reaction volume

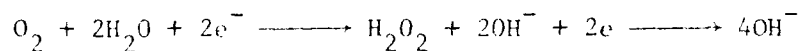
6.22 = millimolar extinction coefficient of NADH at 340 nm

0.01667 = volume of medium in cuvet ($1/3 \times 0.05 \text{ ml}$)

1 = light path (cm)

With medium which contains serum and which has not been incubated with cells, a slight background A_{340} arises which is subtracted from the A_{340} of the test sample. This background averages 0.049 for medium containing 1% serum and 0.155 for medium containing 10% serum. The coefficient of variation for 17 samples at an average A_{340} of 0.155 was 8.9%. The limit of sensitivity for this test is considered to be $\approx 1.2 \text{ mg/100 ml}$ corresponding to an A_{340} of 0.015. Due to the necessity for background subtraction, the practical limit is considerably higher.

We are measuring oxygen in the medium by an Instrumentation Laboratory Model 113 pH/Gas Analyzer. This machine has an electrical accuracy of 0.5% for all scales and an accuracy of $\pm 1\%$ of the full scale $p\text{O}_2$. Oxygen is determined amperometrically by this instrument. This instrument uses a constant polarizing voltage of 0.6V. Reduction of O_2 at the $p\text{O}_2$ cathode occurs according to the equation:



The electron flow is proportional therefore to the amount of oxygen consumed. The electrode is specific for oxygen because it is covered by a polypropylene membrane which is permeable only to gases²⁴. The instrument is calibrated by

zeroing using a gas mixture containing 10% CO₂, 90% N₂. An upper point is set using a mixture of 5% CO₂, 11.82% O₂, and the remainder N₂. The meter is calibrated in the morning and afternoon. Drift during the day has been found to be less than 5 mm Hg and at night less than 7 mm Hg over many days of continuous use.

2.3 ARTIFICIAL CAPILLARIES FOR CELL GROWTH

2.3.1 Choice of Hollow Fiber Units

For many of the cell growth studies, artificial capillary units were purchased from Amicon Corporation. These units, shown in Figure 5a, contain 150 capillaries prepared from polysulfone (Vitafiber[®] 3S100). The nominal protein retention of the lumen surface is 100,000 MW, determined by ultra-filtration under pressure. Molecular weight cutoff for molecules in a diffusion mode may be even higher. The advantage of these capillary units is the fact that they are completely autoclavable (121°C, 10-15 min, 15 lb/in²). Although the shell entry ports were designed with Luer fittings, the fiber bore entry and exit were not; for ease in operation, fittings were added at these points using a short length of silastic[®] tubing. Polysulfone fibers, although autoclavable, have been shown to become less permeable due to binding of serum protein to the lumen wall after continued use with protein. This loss of permeability does not noticeably affect cell growth, however, as some of these units have now been in operation for nearly four months.

However, there are two other potential disadvantages to the use of these capillary units as biological detector prototypes. The polysulfone fiber is highly polar and might adsorb trace components in the medium, thereby lowering the detector sensitivity. The construction of the units will influence the time for the detector to respond to toxin. The extracapillary volume of the Amicon unit is approximately 2.6 ml and the intracapillary volume only 0.27 ml. If toxin is passed through the fiber bore, it must pass through the fiber walls in order to reach the cells on the shell side. The initial toxin-containing medium will therefore be diluted until eventually it displaces the residual medium. Such dilution could interfere considerably with the sensitivity of the test. Also the detection of metabolic response by lactic acid or

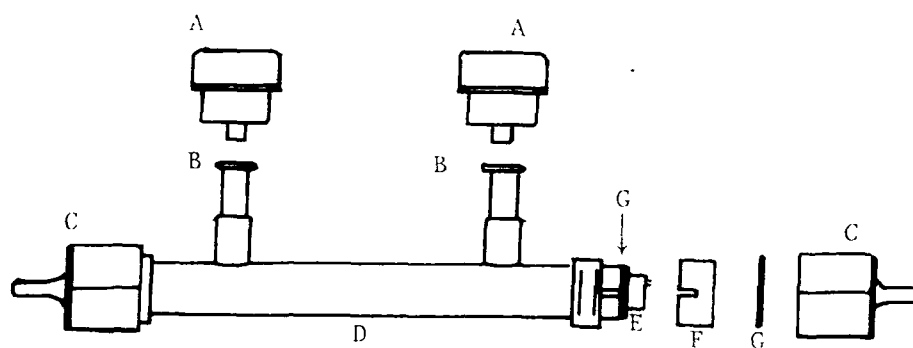


Fig. 5a. Designs of artificial capillary units.

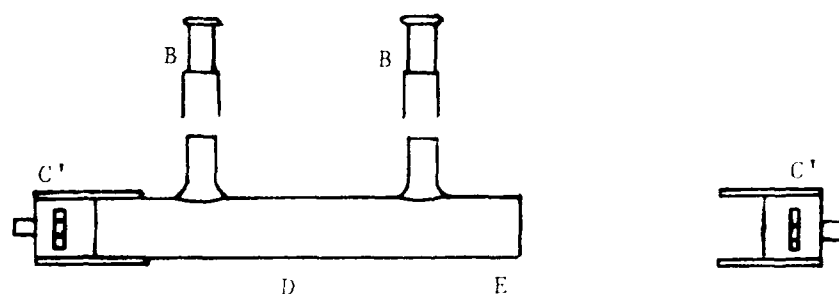


Fig. 5b. Designs of artificial capillary units.

- | | |
|--------------------------|------------------------------|
| A - septum fittings | D - Culture tube |
| B - female Luer | E - capillary entry (potted) |
| C - tubing connector | F - Anti-rotation ring |
| C' - male Luer connector | G - O-ring |

glucose determination might be delayed for the same reasons. These same considerations apply if the toxin-containing medium is directly passed through the shell and therefore is at all times in direct contact with the macrolabeling cells. In this perfusion scheme, the entering medium containing toxin must displace medium in the shell in order to bathe all the cells in the unit. The smaller the shell volume the more rapidly the displacement will take place and the sooner the cells will respond to their new environment.

Hollow fiber units are being developed by GSRI (see Fig. 5b). The properties of these units are compared in Table 1 to those listed for the Amicon 3S100 units. The extracapillary volumes of GSRI units #1 and #2 are seven times and three times that of their respective intracapillary volumes. With the Amicon unit the difference in volume is tenfold. The extracapillary volume of both GSRI units is about four times less than that of the Amicon unit; therefore, for through-shell flow a significantly more rapid response time would be anticipated. The GSRI units, however, have a twofold lower total surface area for cell growth and, therefore, would be expected to support fewer cells than would the Amicon unit. The GSRI units are not autoclavable and are sterilized by either ethylene oxide or 3% formalin. The GSRI units contain a highly permeable hollow fiber made of cellulose acetate (40% acetyl, ASTM visc 25, Eastman Org.) precipitated in water from a formamide/acetone solution. Fiber #1 had a hydraulic permeability L_p (H_2O) at 200 mm Hg pressure of 970×10^{-5} cm/sec atm, fiber #2 a permeability of 747×10^{-5} cm/sec atm and Amicon 3S100, a permeability of 240×10^{-5} cm/sec atm.

In our experiments both GSRI units and the Amicon 3S100 support the growth of BHK and L929 cells. Final cell populations up to 60×10^6 cells have been achieved for both units, so performance must be judged by their relative abilities as toxin detectors.

2.3.2 Production of GSRI Units

Hollow fiber units are prepared from polycarbonate tubes (70 mm ID) 8 cm long. Sidearms to provide entry to the shell are attached at 2 cm from each end (Fig. 5b). The effective growth area is 5.5 to 6.0 cm. The fiber ends are constricted with a suture tie and then sealed in the tubes with RTV-11[®]

Silicone rubber (General Electric) or Vorite-689 287-700 (Polycin 942[®]), a urethane polymer from Industrial Chemicals Division of NL Industries. With the former sealing agent, the fibers can be left wet during the process but with the latter the fibers are immersed in 100% glycerol and allowed to dry before potting. The glycerol treatment and drying does not appreciably affect the molecular weight permeability of the fibers. With the urethane potting, leaks between the shell and bore fluid are seldom experienced but with the silicone potting such leaks can develop after repeated use. All unit entry ports are fitted with Luer-loc adapters by means of Tygon[®] tubing (see Fig. 5b). Such an arrangement allows easy entry to the shell compartment and versatility in placing the unit into different perfusion circuits. Before use the units are tested for leaks by separately passing air through the fibers and the shell while immersing the unit in water. During this operation the second unit end or shell port, respectively, is closed by a rubber septum. Rapid bubbling is evidence of leaks; in such cases the unit is not used. More than two-thirds of the units so far produced passed this test.

2.3.3 Washing and Sterilization of Hollow Fiber Units

After assembly of the fibers into the units, the units are washed with running water for several days and then are soaked in a 10% ethanol-water solution. The units are also washed for several hours by ultrafiltration of water through the fibers. This extensive washing is intended to remove traces of the formamide used as a solvent for the cellulose acetate. After washing, the GSRI units are allowed to sit in a 2.5-3.0% formalin solution overnight; care is taken to avoid entrapment of air in the shell or fibers. Following the formalin sterilization, the unit is fitted with ethylene oxide-sterilized Tygon[®] fittings containing Luers (see Fig. 5b) and connected into a sterile (autoclaved) perfusion circuit as shown in Figure 1. The circuit is flushed with sterile water for three days, 10% ethanol-water for one day, and medium containing serum for one day prior to cell planting. In some cases the units (containing fittings) were sterilized by ethylene oxide for 24 hours and then washed as above. The Amicon units were steam sterilized before use, and no washing was necessary. For reuse of units after performing a study,

the cells were removed by treatment with 0.25% trypsin- 10^{-2} M EDTA (ethylene diamine tetraacetic acid). The units were then washed in water, retreated with trypsin-EDTA, rinsed and then sterilized as above. Reused units, whether manufactured by Amicon or by GSRI, showed no loss in their original ability to support thriving cell cultures.

TABLE I

PROPERTIES OF DIFFERENT ARTIFICIAL CAPILLARY
UNITS FOR CELL CULTURE

	Amicon 3S100	GSRI #1	GSRI #2
Capillary type	polysulfone	cellulose acetate	cellulose acetate
Potting	epoxy, with silica	silastic	silastic
Number of fibers	150	150	75
ID	250 μ m	109 μ m	275 μ m
OD	350 μ m	200 μ m	475 μ m
Total area, fiber surface	94 cm^2	57 cm^2	48 cm^2
Effective fiber length	5.7 cm	6.0 cm	6.0 cm
Intracapillary volume	0.27 ml	0.0855 ml	0.269 ml
Extracapillary volume	2.6 ml	0.6 ml	0.8 ml
Molecular weight permeability	N/A	permeable to albumin	permeable to albumin

2.3.4 Planting Cells on Artificial Capillary Units

Units are planted by injection of the cells into the shell volume with a 1-cc syringe, while a second syringe is used to withdraw the air. During this operation flow of medium to the unit is stopped and the entry and exit tubing are clamped. The cells are then rinsed back and forth between the syringes several times to allow them to penetrate between the fibers. Flow is started 10-20 min from initial planting, and 30-50 min after trypsinization of

flask cultures. After 30 min to 2 hr the unit is turned over to allow cells which settle on the bottom (the polycarbonate shell) rather than on the fibers to fall back onto the fibers. From 5 to 30×10^6 cells are routinely planted on each unit. Additional medium, if necessary, is added to fill the shell, and the shell entry ports are sealed.

2.4 OPTIMIZATION OF THE ARTIFICIAL CAPILLARY UNITS AS A BIOLOGICAL DETECTOR

In order to satisfy the requirement that the MUST detector be capable of a rapid response upon exposure to an acute toxin, two conditions must be satisfied: (1) The cell culture system must be sufficiently sensitive to toxin to allow the cells to alter their metabolic activity within minutes after exposure and (2) the metabolic rate change must be detectable in this time by a simple (preferably in-line) sensitive assay for glucose, oxygen, or lactic acid in the medium in contact with the cells.

2.4.1 Optimization for Lactic Acid and pH Determination

We tested two different operational modes which allow buildup of detectable levels of lactic acid in the medium and depletion of detectable levels of glucose from the medium using a once-through flow. When these modes were used with medium not containing Hepes, the medium pH also decreased significantly. In the first mode of operation the perfusion medium was allowed to flow through the unit at a rate sufficiently slow to allow significant glucose depletion and lactic acid buildup in the medium by the cells in a once-through flow. The pH of the medium (not buffered with Hepes) changed by over 0.5 pH units between inlet and outlet when a flow rate of 0.06 ml/min or slower was used. In the second mode of operation, a fast flow rate was alternated with no flow. The second mode was tried because oxygen starvation probably occurs during periods when flow is halted, but would be reversed during periods

of fast flow: therefore such an operational mode might be less harmful to continuous healthy cell maintenance than a system in which the cells are constantly in oxygen debt. As will be seen later, if the "detector" is oxygen depletion, a continuous flow system for a biological detector is achievable without such considerations.

To test these two modes of operation we used a unit containing BHK cells (GSRI #1 fibers) on which cell growth had been followed for more than two weeks. No contamination by microorganisms was detectable by periodic culture of waste medium samples in Brain Heart Infusion broth or in Thioglycollate medium. Also no fungal growth was visible. The unit was fed by medium circulated by the aid of a variable speed (Buchler polystaltic) peristaltic pump. Flow rate was varied on the same unit from 0.04 to 2 ml/min and samples of medium were collected from a stopcock placed at the exit from the shell (F in Fig. 1). The variation in pH and lactic acid with flow rate is shown in Figure 6. As can be seen at slow flow rates (<0.1 ml/min) for this unit, the outlet is more than 0.5 units more acid than the inlet pH (but still well within a range suitable for cell culture). Most of this change is presumably due to the excretion of lactic acid into the medium by the BHK cells; at 0.1 ml/min this amounts to almost 12 $\mu\text{g}/\text{min}$. In Figure 7 we have plotted the concentration $C(\text{mg}/100\text{ml})$ versus $1/Q$ (ml/min) where Q is the flow rate. The excretion rate (U) is equal to $Q \cdot \Delta C$ where ΔC is the difference between the inlet and outlet concentrations in mg/100 ml. If the excretion rate is not flow rate-dependent, then this graph should give a straight line with a slope, U . As can be seen in Figure 7, at slow flow rates ($Q < 0.2$ ml/min) the metabolic rate decreases somewhat.

In a separate experiment we studied the effect of prolonged exposure to low flow rates on cellular metabolic activity. The measurements given in Table 2 were made over 3 days at each of the three flow rates. Again, established cultures of BHK cells on GSRI fiber units were used. The metabolic rate as determined by lactic acid excretion is flow rate-dependent, but this rate does not decrease over the three-day period. These findings indicate that even prolonged exposure to maintenance conditions in which some portion of the cell population is presumably anoxic does not cause a rapid cell die-off.

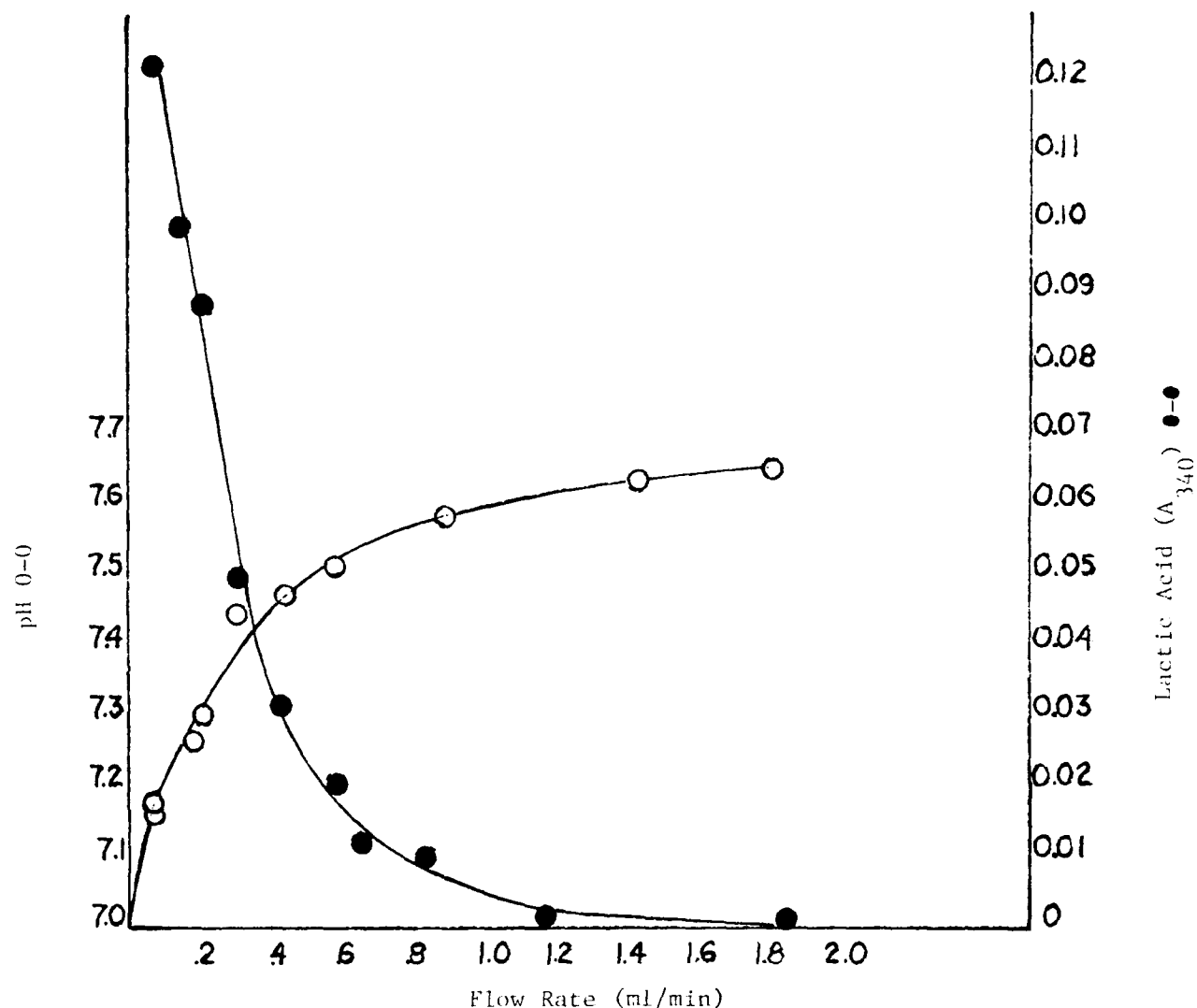


Fig. 6. Variation of pH and lactic acid with flow rate.

An established culture of BHK/21 cells on a GSRI capillary unit was perfused through the bore at different rates by DMEM with 10% fetal calf serum. The bicarbonate in the medium was 0.35 g/L. The medium leaving the unit was collected, (1 ml) placed in plastic tubes and after the final point was taken, all samples were assayed for pH. The pH of the medium entering the unit was 7.4 but, due to CO_2 loss, rose to 7.6 over this time. The medium was allowed to perfuse the culture unit for 30 min at each speed before an aliquot was taken.

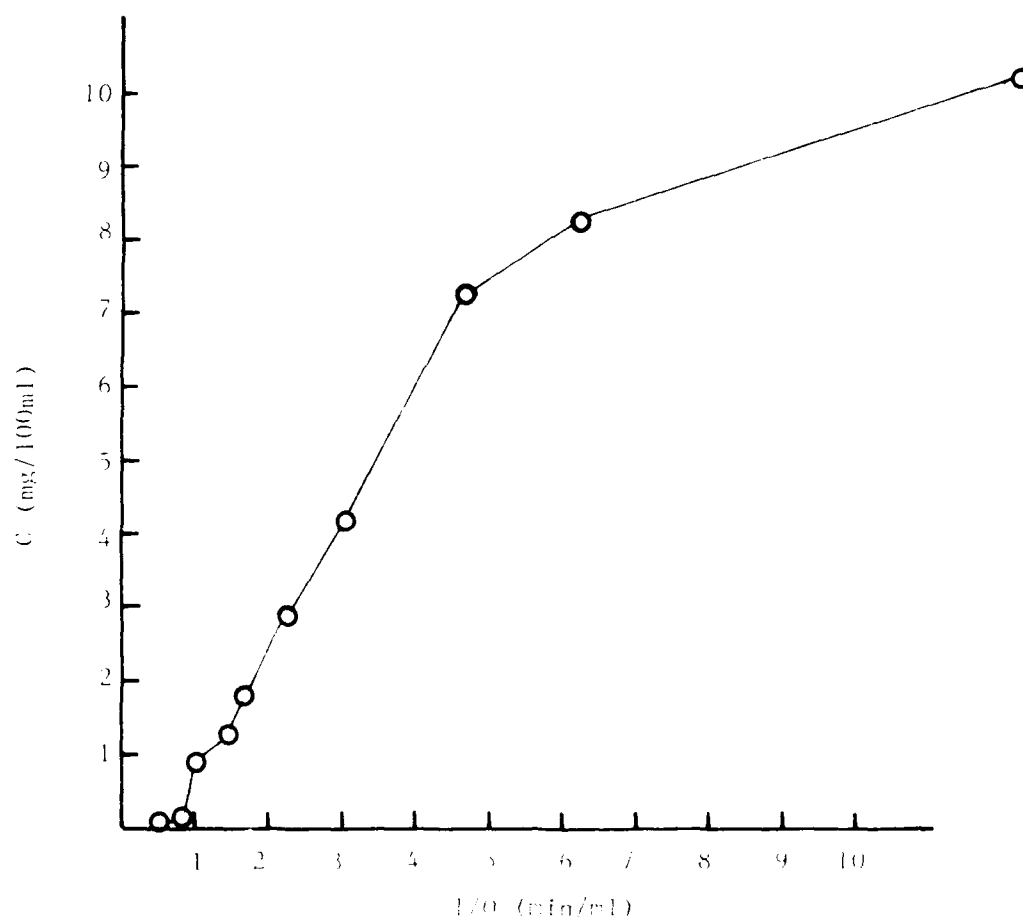


Fig. 7. Dependence of lactic acid excretion by BHK cells on perfusion flow rate; C = lactic acid concentration, mg/100 ml
 Q = flow rate ml/min.

The BHK/21 cells were maintained as described in the legend to Fig. 6. The utilization rate (U) is defined at each point on the curve by the relation $C = 1/Q \cdot U$. At flow rates slower than 0.2 ml/min (for this specific capillary culture unit), U is flow rate dependent. At flow rates greater than 0.2 ml/min, U is flow independent.

TABLE 2

EFFECT OF PROLONGED EXPOSURE TO
SLOW FLOW RATES ON CELL METABOLISM

Time (hr)	Lactic Acid Utilization Rate		
	(mg/min) $\times 10^2$		
	Flow Rate(a) 0.18 ml/min	Flow Rate(b) 0.12 ml/min	Flow Rate(c) 0.06 ml/min
0	2.7	3.1	1.9
12	2.9	3.1	1.9
24	2.9	3.2	1.9
36	2.9	3.0	1.9
48	2.9	3.1	1.9
60	2.9	3.0	2.0
72	2.9	3.0	2.0

Presumably the adenosine triphosphate (ATP) production necessary for cellular processes arises only or mainly from glycolysis under these conditions, whereas when oxygen is available, relatively more ATP arises from respiration. These observations were made by Gregg, et al.²⁵ for cells in suspension; a higher glycolytic rate was found for cells under anaerobic conditions than was found under aerobic conditions for growth.

Results similar to those obtained with a slow flow method are seen with the stopped flow method. The medium sampled from within the tubing bore for the periods of time shown in Figure 8 probably includes some medium that was not in contact with the cells. Therefore, the actual changes are probably substantially greater than those values indicated. Sampling was performed by allowing the medium within the shell and fiber bore to stagnate for the periods of time indicated. This medium was then withdrawn by syringe. Between each stopped flow period, medium flow at 3 ml/min was resumed for 10 min.

The amount of lactic acid excreted is directly proportional to the length of time the medium is in contact with the cells. This relationship indicates that under these temporary anaerobic conditions, metabolic rate was not significantly slowed.

The pH values were determined after the samples were placed in test tubes. No attempt was made to prevent equilibration of the sample with the room atmosphere. Thus, in all cases the measured pH values are higher than the actual pH of both the initial medium and the spent medium due to loss of CO₂ from the medium on standing. An in-line pH electrode would provide a more sensitive measure of the actual differences obtained. In-line methods will be carefully tested during our second year of research effort on development of a biological detector.

2.4.2 Optimization for Oxygen Determination

During the performance of the studies described in section 2.4.1, an oxygen detector was not available. Since that time, we obtained the use of an IL 113-01[®] instrument used clinically for blood gas determinations. This instrument has been incorporated into the perfusion schemes shown in Figure 4. The oxygen tension of the input medium is 150 mm Hg. We tested the effect of slow perfusion rates on output oxygen content of the medium. The culture

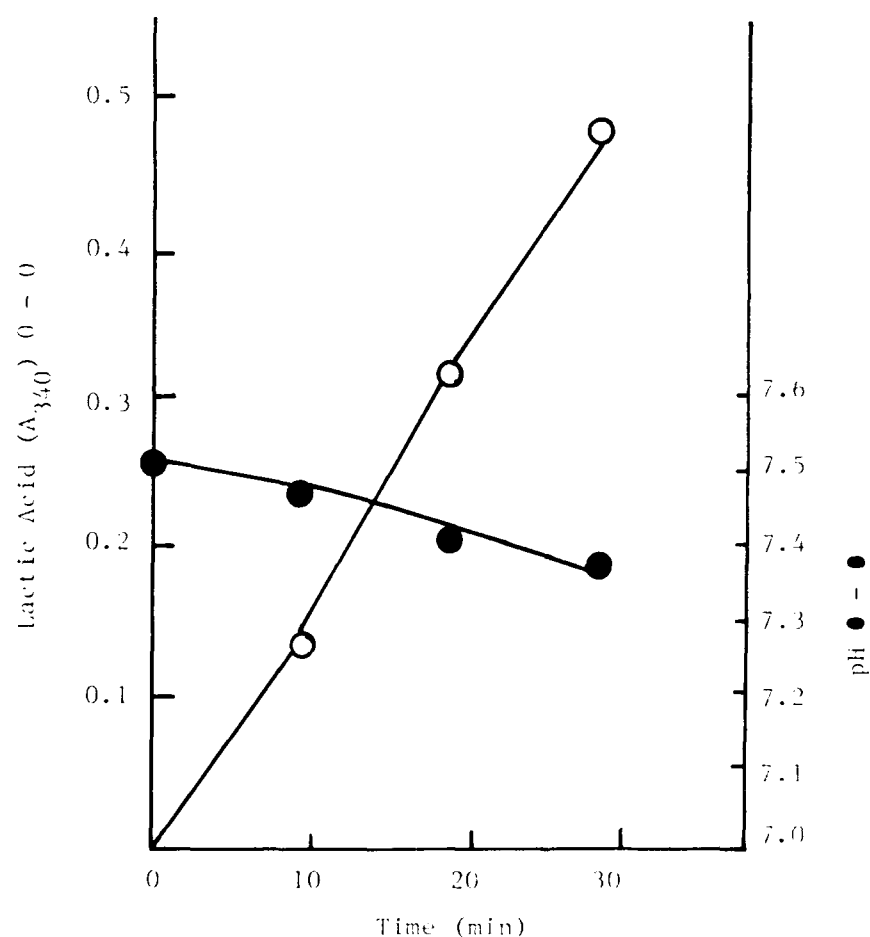


Fig. 8. Exposure of cells to medium for different periods of time by stopping flow.

An established culture of BHK cells on GSRI #1 unit was perfused by medium at 2 ml/min. Flow was then stopped for the times indicated on the graph abscissa. After these times, one ml of medium on the unit side was removed by a syringe attached to a stopcock F (see Fig. 1). Assays were performed after all samples were collected. Between each stopped flow period, flow was resumed at 3 ml/min for 10 min.

tested in these studies contained BHK cells on a GSRI unit. The cells were in culture for 2 weeks prior to this study and had reached a plateau in metabolic rate as indicated from glucose and lactic acid determinations made over this period of time.

In Figure 9a is plotted the variation of outlet pO_2 with perfusion flow rate Q , and in Figure 9b, variation of ΔpO_2 with $1/Q$. As with lactic acid the points in the plot in Figure 9b can be fitted to a straight line for flow rates 0.2 ml or higher; a straight line indicates a constant utilization rate at these perfusion rates. With slower perfusion, the cells use almost all the medium oxygen and utilization rate seems to slow slightly.

In recent studies we have attempted to measure lactic acid excretion, glucose utilization and pO_2 depletion simultaneously on the same detector unit. We chose a unit containing L929 cells whose outlet pO_2 was 70 mm Hg at a flow rate of 0.2 ml/min. Since the inlet pO_2 was 150 mm Hg, the utilization over this time was 80 mm Hg corresponding to $\sim 3 \mu l O_2/ml$ of medium. The utilization rate is equal to $(0.2 \text{ ml/min}) \times 3 \mu l/min$ or $0.6 \mu l/min$ for this unit. Using the known utilization rate of oxygen by L929 cells²⁶ of $7.0 \mu l/hr/10^6$ cells (obtained for unperfused cultures), we calculate that this unit contains 5.1×10^6 cells. Such a cell population would be expected to have a glucose utilization rate of $\sim 1.07 \mu mol/hr$ and a lactic acid excretion rate of $\sim 2.04 \mu mol/hr$ ¹. At a flow rate of 0.2 ml/min these utilization rates would correspond to a lactic acid concentration of $0.170 \mu mol/ml$ and a glucose concentration of $5.51 \mu mol/ml$ and a ΔOD for the colorimetric test of 0.019 and 0.006, respectively. These ΔOD are too low to be significant in the colorimetric tests. Therefore, we would not expect to be able to measure glucose and lactic acid at flow rates which would allow any outlet pO_2 . Experimentally, we have verified this conclusion. Samples were collected from a unit containing L929 cells whose effluent oxygen content was 5 mm Hg at a flow rate of 0.35 ml/min. Under these conditions aliquots of effluent and reservoir medium were sampled and their lactic acid and glucose content were measured. The difference detected (<0.01 OD unit) was not significant.

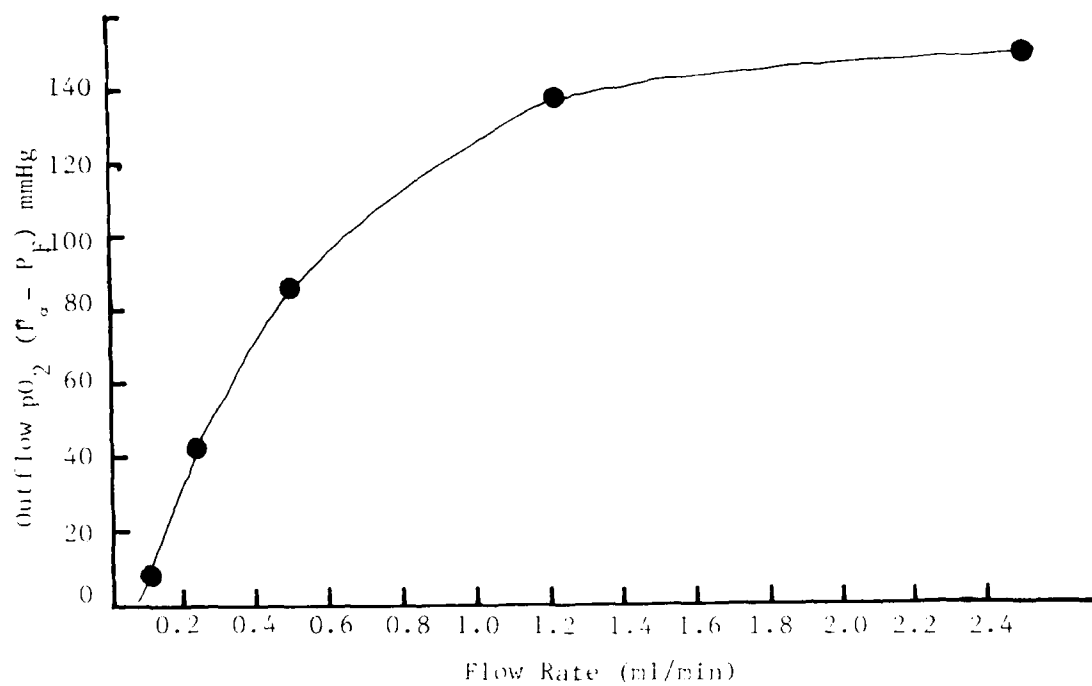


Fig. 9a. Variation of pO₂ with flow rate.

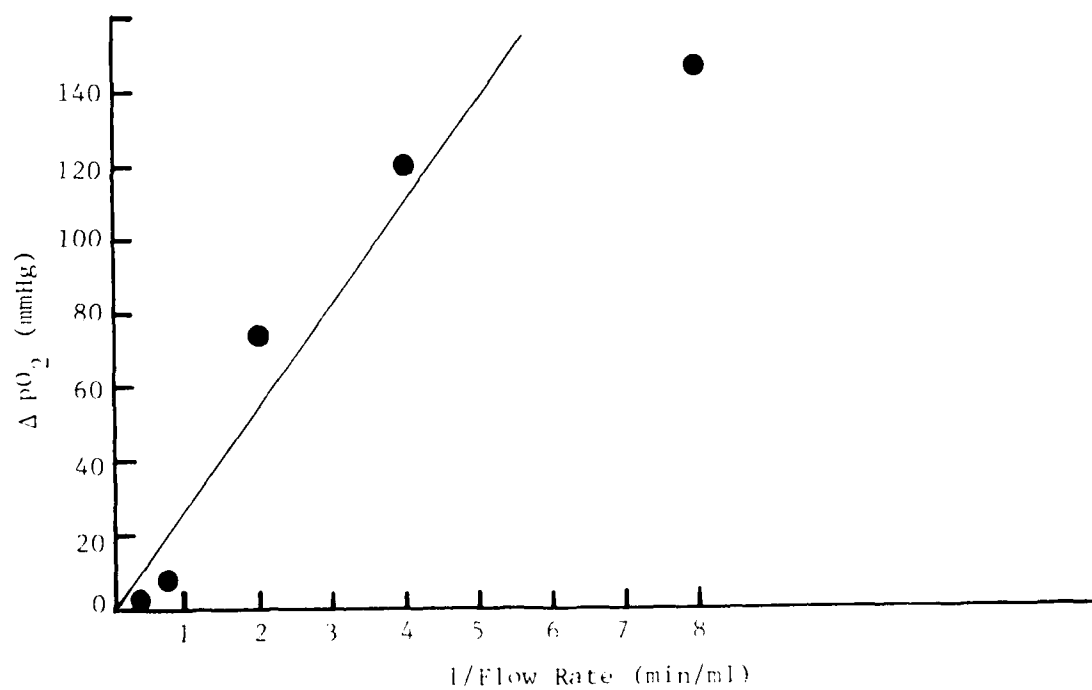


Fig. 9b. Variation of utilization rate with flow rate.

Fig. 9a. Variation of pO_2 with flow rate.

An established culture of BHK/21 cells on an Amicon 3S100 Culture Unit was perfused through the bore of the fibers and then to an oxygen meter (Fig. 4a). Oxygen tensions were continuously recorded on a Sargent Chart Recorder and the values given were after no further change in the tension at each flow rate was found.

Fig. 9b. Variation of utilization rate with flow rate.

Dependence of the oxygen tension on flow rate. With rates faster than 0.2 ml/min essentially no variations in oxygen utilization with flow is found, but at slower rates (in accord with the lactic acid results) a decrease in oxygen metabolism was observed.

2.5 TOXICITY STUDIES USING ARTIFICIAL CAPILLARY UNITS

2.5.1 Toxicity Studies on Cells in a Perfusion Circuit

Our initial studies were performed using a culture unit fed by 25 ml of medium in a reservoir (Fig. 1). The medium was allowed to flow through the unit at 8.5 ml/min and returned to the reservoir. Aliquots (0.5 ml) were withdrawn at hourly intervals and assayed for lactic acid and glucose content. Normal and toxin-inhibited metabolic rates for L929 cells under such a system are shown in the curves in Figure 10. As can be seen, significant concentration changes for lactic acid and glucose are obtained by assaying at hourly intervals. Five time points were chosen for convenience in doing the measurements as well as to minimize the contact time of the cells with toxin. Experiments were performed on the same culture unit, one metabolic rate curve per day. After each challenge with toxin the return to normal or near normal metabolic activity by the cells was assessed on the following day by the procedure described above. Before a new dose of toxin was administered, normal or nearly normal metabolic activity was established. In this study, dinitrophenol toxicity was tested prior to iodoacetate and a return to normal metabolic rate was found. In these experiments, toxin (iodoacetate [1 ppm] and dinitrophenol [2 ppm]) was added to the reservoir containing 25 ml of DMEM supplemented with 1% FCS. The toxin-containing medium was circulated for 10 min at the above rate to insure mixing with the residual medium in the tubing and culture unit; then 0.5 ml aliquots were removed at the indicated times. During this experiment pH was not controlled by Hepes; therefore, the leveling off of the curves may in part reflect metabolic inhibition due to pH change at the later times. Final pH was approximately 6.9.

The slopes of lines drawn from time, $t=0$ to $t=2$ hr in Figure 10 can be used to calculate the metabolic rate for the uninhibited and toxin-treatment conditions. Uninhibited cells utilize glucose at the rate of $0.29 \mu\text{mol/min}$ and excrete lactic acid at the rate of $0.17 \mu\text{mol/min}$. For cells incubated with 2 ppm dinitrophenol the rates are 0.17 and $0.15 \mu\text{mol/min}$, respectively. For cells treated with 1 ppm iodoacetate, the rates are 0.064 and 0.11, respectively.

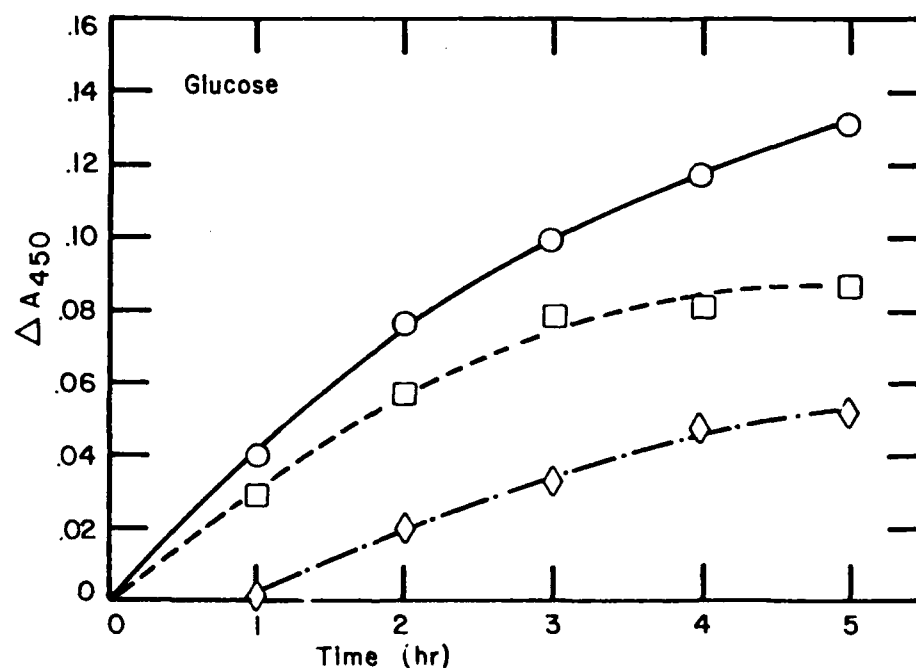
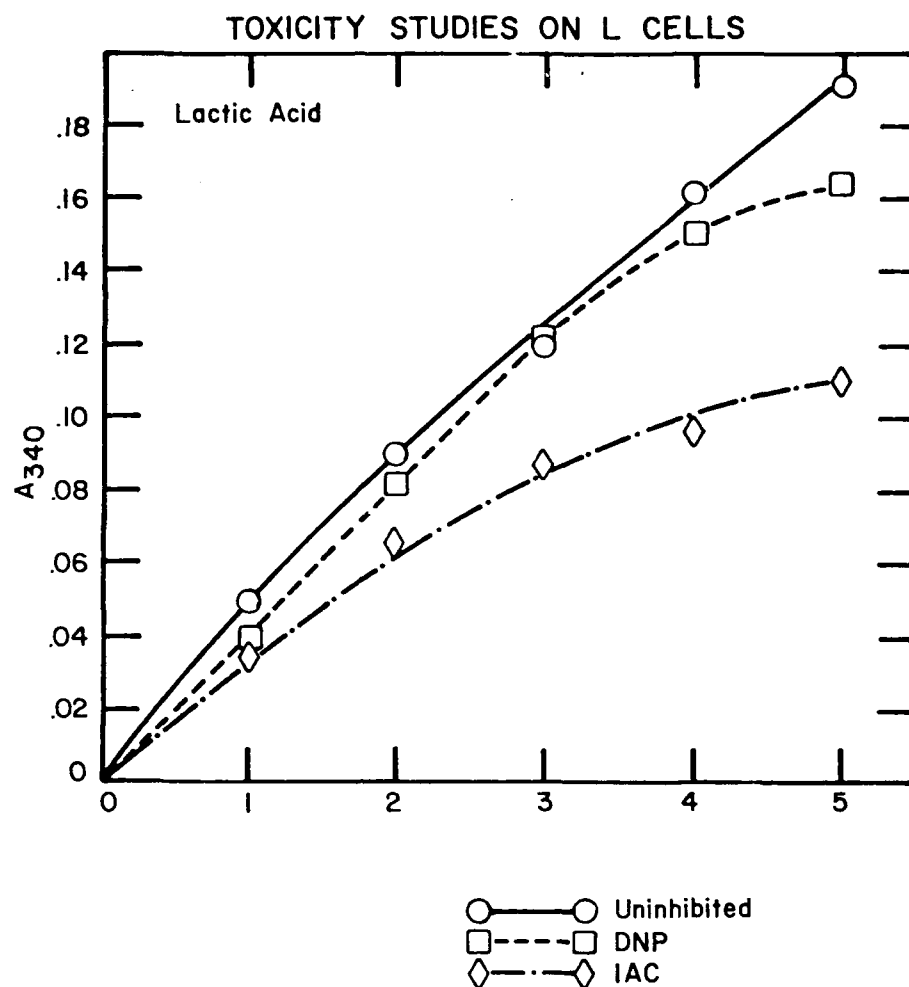


Fig. 10. Change in lactic acid and glucose metabolism of L929 cells after treatment with dinitrophenol (DNP) and Iodoacetate (IAC). 34

Fig. 10. Change in lactic acid and glucose metabolism of L929 cells after treatment with dinitrophenol (DNP) and iodoacetate (IAC).

The L929 cells were maintained by perfusion as in Fig. 1 with a reservoir containing 25 ml DMEM + 10% FCS with or without toxin. At the hourly intervals shown 0.5 ml aliquots of the medium were withdrawn from the reservoir and assayed for lactic acid and glucose. The conditions of testing were as described in the text. At the conclusion of the iodoacetate study, $\sim 50 \times 10^6$ cells were recovered.

From Figure 10 a metabolic rate decrease caused by toxin is detectable even at the initial time points. Therefore, at least for the metabolic toxins, iodoacetate and dinitrophenol, rapid response could be detected if the detector could be made more sensitive. Two methods of achieving a more rapid detector response are evident from consideration of the curves in Figure 10 and the mode in which the detector was operated. If, instead of 25 ml in the reservoir flask only 1 ml could be circulated, the detection time could be decreased to as little as 12 min. A second method of decreasing the response time in such a circuit requires a proportionate increase in the sensitivity of the assay method. That is, by a tenfold increase in the sensitivity of the lactic acid assay (an achievable objective using a fluorimetric method), aliquots taken from $t=0$ to $t=1$ hr would have sufficient lactic acid to give a significant optical density. Thus, the inhibited and uninhibited metabolism of the cells could be compared. A third method would be to increase the utilization rate/unit time by increasing the number of cells on the unit.

2.5.2 Toxicity Studies on Cells Using Once-Through Perfusion

2.5.2.1 Lactic Acid Excretion Studies

A preferred system would allow media to flow from a reservoir through the unit and then to waste. For such an approach to be feasible, the flow rate must be slow enough that inordinate amounts of medium are not required to perfuse the unit and so that buildup or depletion of metabolites in the medium is large enough to be detected with accuracy. Furthermore, for the MUST detector, a 5-30 min response time was set as a desired goal. We have been testing the most highly toxic components, studied by other toxicity criteria in the MUST program, namely, *o*-toluidine, N,N-diethyl-m-toluamide and phenol, rather than dinitrophenol and iodoacetate. In addition, potassium cyanide was used as an example of a well-known acute toxin. The dose which inhibits growth to 50% of the control value (EC_{50}) found for these toxins are 26, 119 and 50 ppm, respectively²⁷. In these tests, BHK cells were first grown in duplicate capillary culture units in the perfusion circuit of Figure 1 until a plateau in metabolic activity was reached. The unit was then placed

in the perfusion scheme shown in Figure 2a and perfusion with 1% serum-containing medium was begun at a flow rate of 0.120 ml/min. This medium was allowed to go to waste after perfusing the unit. Each challenge with toxin was begun by flushing the old medium from the tubing at 1 ml/min for 10 min using the toxin-containing medium. Then operation at 0.05 ml/min was initiated in order to maximize the lactic acid response. After 5, 10, 15, 20, 25, 30, and 35 min, the medium which had flowed through the fibers was collected from a syringe attached to a three-way Luer stopcock located immediately after the culture unit. Before and after each challenge with toxin-containing medium, the same medium without toxin was allowed to pass through the fibers, and samples were collected as above to establish a "normal" metabolic rate in the absence of toxin. The lactic acid content of the samples was then determined. These results and the average utilization rates are given in Table 3 for o-toluidine and Table 4 for N,N-diethyl-m-toluamide. The results are averages of the two determinations on two different culture units containing BHK cells and maintained identically. Deviations were less than 10%.

In some cases, the early time points listed in these tables show an upward drift due to equilibration of the lactic acid with the dead volume in the shell side of the unit. When medium was introduced through the bore at high flow for about 10 min, most of the lactic acid in the shell volume was dialyzed out, while the toxin diffused in. After this exposure, the flow was slowed to permit measurement; during this analysis period, not only the bore volume but also the shell volume increases in lactic acid concentration. When the fast "maintenance" flow is restored (this fast flow was also used to supply oxygen to all the cells on the culture units), the accumulated lactic acid in the shell again dialyzes out. It takes about 15 min to reach steady state equilibrium when the Amicon units, with their 2.6 ml dead volume, are used (as they were for these experiments). After a steady state level was reached, further drift did not occur over the measurement time course.

No oxygen meter was available to us when the above experiments were performed. Thus, we did not know at that time, that the outlet oxygen content of the medium at this flow rate was probably zero and that some portion of the cell population was in oxygen debt. These conditions have been subsequently confirmed by use of an oxygen meter. Since the control unit (no toxin) was

Table 3

Effect of o-Toluidine on BHK Cells: Lactic Acid
Excretion ($\mu\text{mols/hr}$)

concentration of o-toluidine

<u>time</u> <u>(min)</u>	<u>0 ppm</u>	<u>1 ppm</u>	<u>0 ppm</u> <u>(Repeat)</u>	<u>10 ppm</u>
5	5.91	4.80	6.84	0.96
10	7.56	5.22	6.87	0.96
15	9.63	5.49	6.87	1.11
20	10.32	5.55	6.45	1.23
25	10.71	5.76	6.60	1.38
30	10.71	5.91	7.02	1.38
35	10.71	6.18	6.45	1.23

Table 4

Effect of N,N-diethyl-m-toluamide on BHK Cells:

Lactic Acid Excretion ($\mu\text{mol/hr}$)concentration of N,N-diethyl-m-toluamide

<u>time</u> <u>(min)</u>	<u>0 ppm</u>	<u>1 ppm</u>	<u>10 ppm</u>	<u>100 ppm</u>	<u>1000 ppm</u>
10	5.76	2.88	3.72	2.76	1.23
15	7.95	3.72	5.37	3.03	1.50
20	8.22	5.49	5.64	3.03	1.68
25	8.25	6.18	6.06	2.88	1.68
30	8.52	6.72	6.06	2.88	1.92
35	8.79	6.73	6.18	3.03	2.07

operated under flow conditions identical to those for toxin-perfused units, results can still be compared, unless it is found that the slow flow induces a metabolic abnormality, and that this abnormality combines with the toxic effect to vitiate the response. We feel that the slight lowering of metabolic rate when either N,N-diethyl-m-toluamide or o-toluidine is perfused through the culture unit is an indication of a cellular toxic response to these compounds.

More sophisticated experiments are now being performed to elicit a toxic response of cells maintained in a once-through perfusion system. In these studies described below oxygen uptake is monitored continuously.

2.4.2.2 Oxygen Utilization Studies

The effect of various toxins (cyanide, o-toluidine, iodoacetate) on the oxygen utilization rates of cells on hollow fibers was measured using the once-through perfusion modes of Figures 4a and 4b. Medium contained 1% calf serum and was buffered by Hepes (initial pH 7.3). The medium with toxin and control medium without added toxin were allowed to flow through the artificial capillary unit at 0.25 ml/min. The first series of experiments used the perfusion scheme of Figure 4a through the fiber bore; in the second series, medium was perfused through the shell as shown in Figure 4b. In the later experiments, prior to perfusion, the shell medium was replaced with the same medium used for the perfusion in order to demonstrate the real-time necessary to elicit a cellular metabolic response following toxin exposure.

Toxins were iodoacetate at 100 ppm, cyanide at 6.5 and 0.65 ppm and o-toluidine at 100 ppm and 28 ppm. With o-toluidine no response of either L929 or BHK cells was seen over 3 hr when medium carrying the o-toluidine was allowed to flow through the capillary fiber bore. However, when the medium in the shell was replaced with o-toluidine, at 28 ppm and 100 ppm, a decline in oxygen utilization occurred within 10 min (Figs. 11 and 12a) although simple replacement of the shell medium (Fig. 12b) did not alter the oxygen utilization rate of the cells significantly. A similar decrease in oxygen utilization is found when cyanide-containing medium is either perfused through the shell or through the bore, shown in Figure 13 and Figure 14, respectively. The response to cyanide is much slower when the shell medium is not replaced (Fig. 13) than

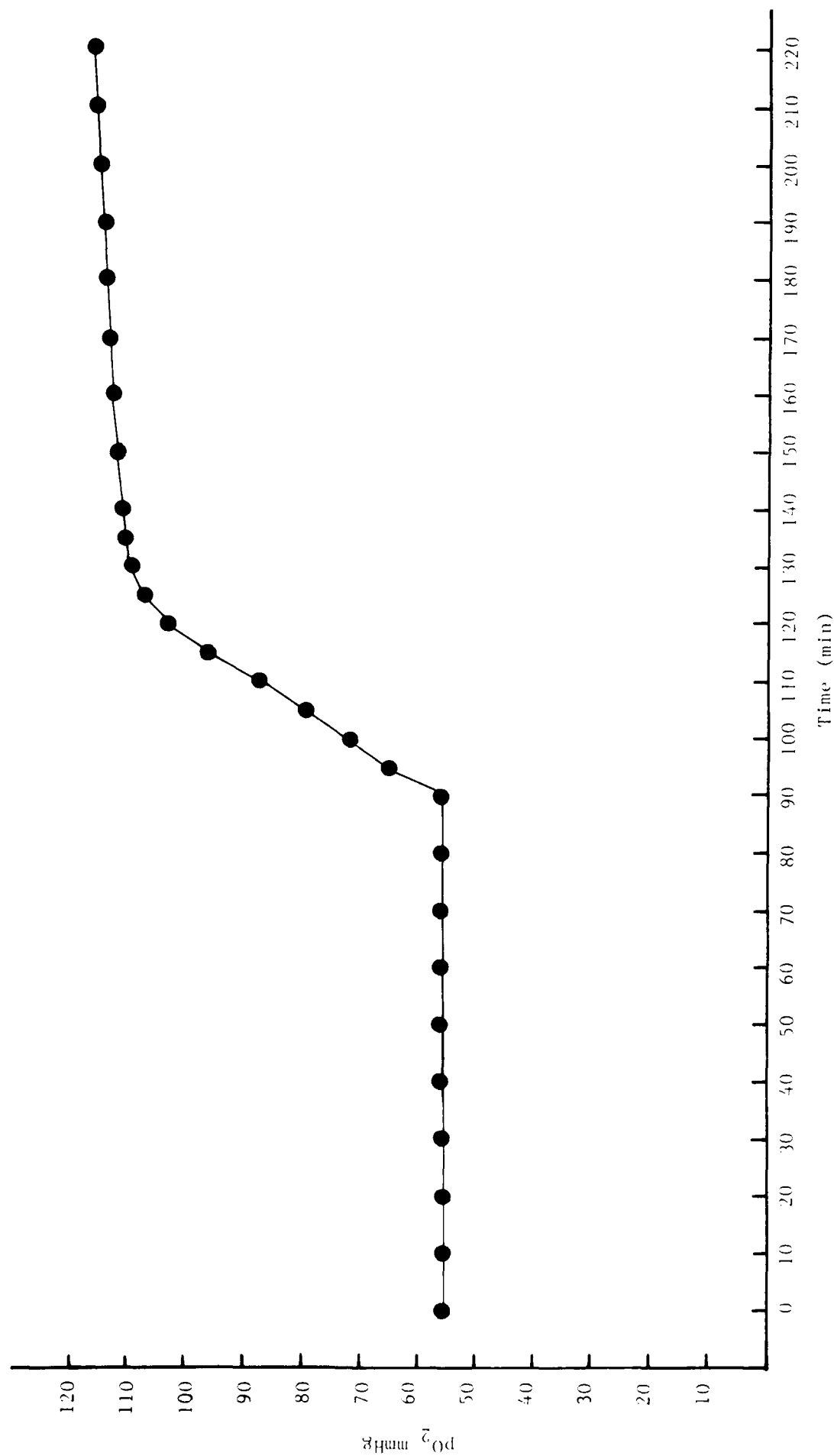


Figure 11. Change in oxygen utilization rate of 1929 cells after exposure to 28 ppm *o*-toluidine. 1929 cells on an Amicon 3S100 unit were perfused at 0.27 ml/min with DMEM, 1% GS, 20 mM Hepes and antibiotics using the perfusion mode of Fig. 4a. At 90 min the medium in the unit shell was replaced by the same medium containing 28 ppm *o*-toluidine and perfusion at the same flow rate was continued.

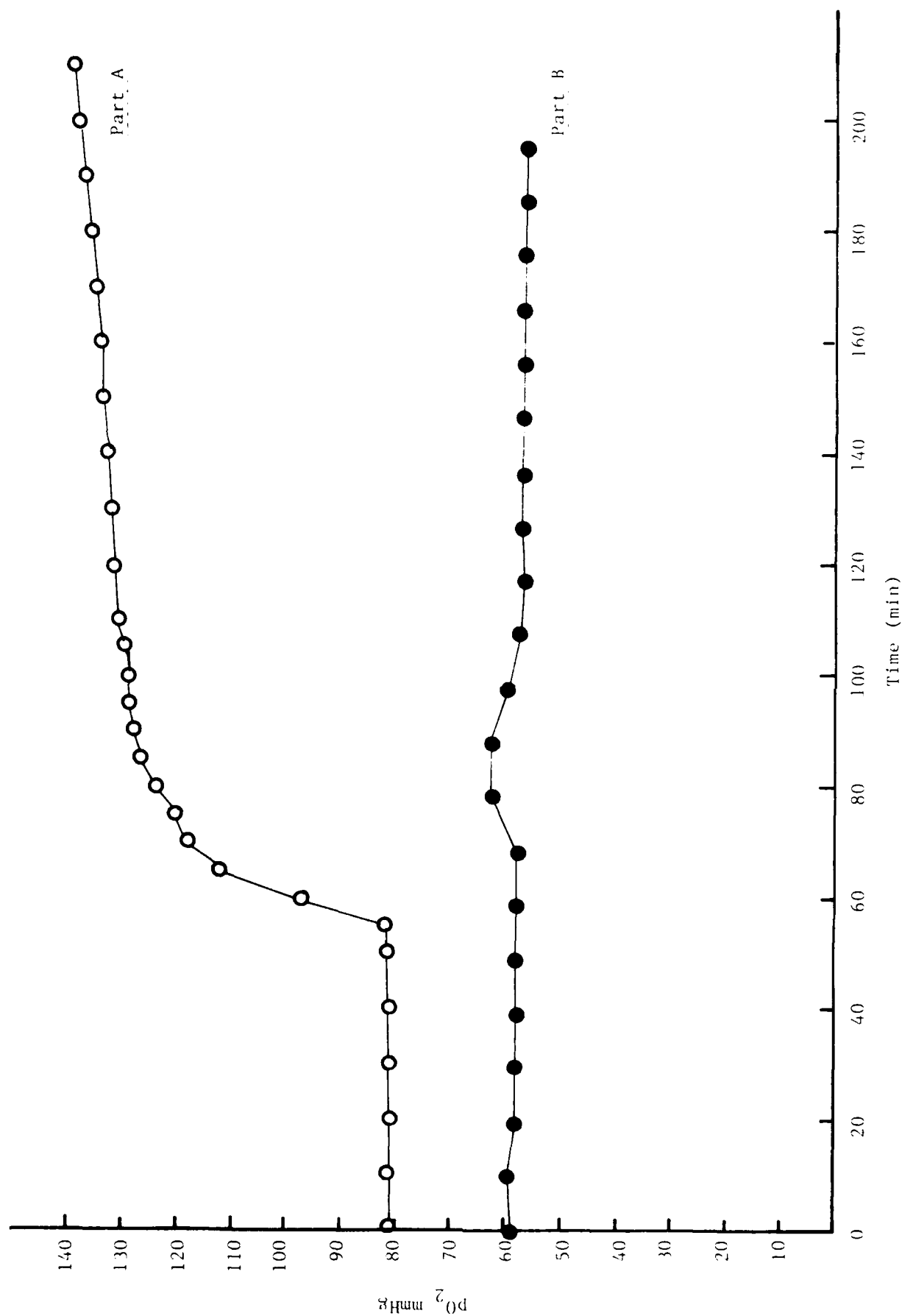


Fig. 12. Change in the oxygen utilization rate of BHK cells on an artificial capillary unit after exposure to 100 ppm o-toluidine.

Fig. 12. Change in the oxygen utilization rate of BHK cells on an artificial capillary unit after exposure to 100 ppm *o*-toluidine.

Part A: BHK cells on an Amicon 3S100 unit were perfused at 0.25 ml/min by medium containing 100 ppm *o*-toluidine, using the perfusion mode shown in Fig. 4a. The extracapillary medium was replaced by medium containing *o*-toluidine at 50 min and then perfusion was continued.

Part B: Illustrates that change of the extracapillary medium does not significantly affect the oxygen utilization rate. The medium without was changed at 70 min. By 100 min the utilization rate of oxygen by the cells had returned to the prechange level, whereas when medium with toxin was added as in Part A, a decrease in O_2 utilization was found.

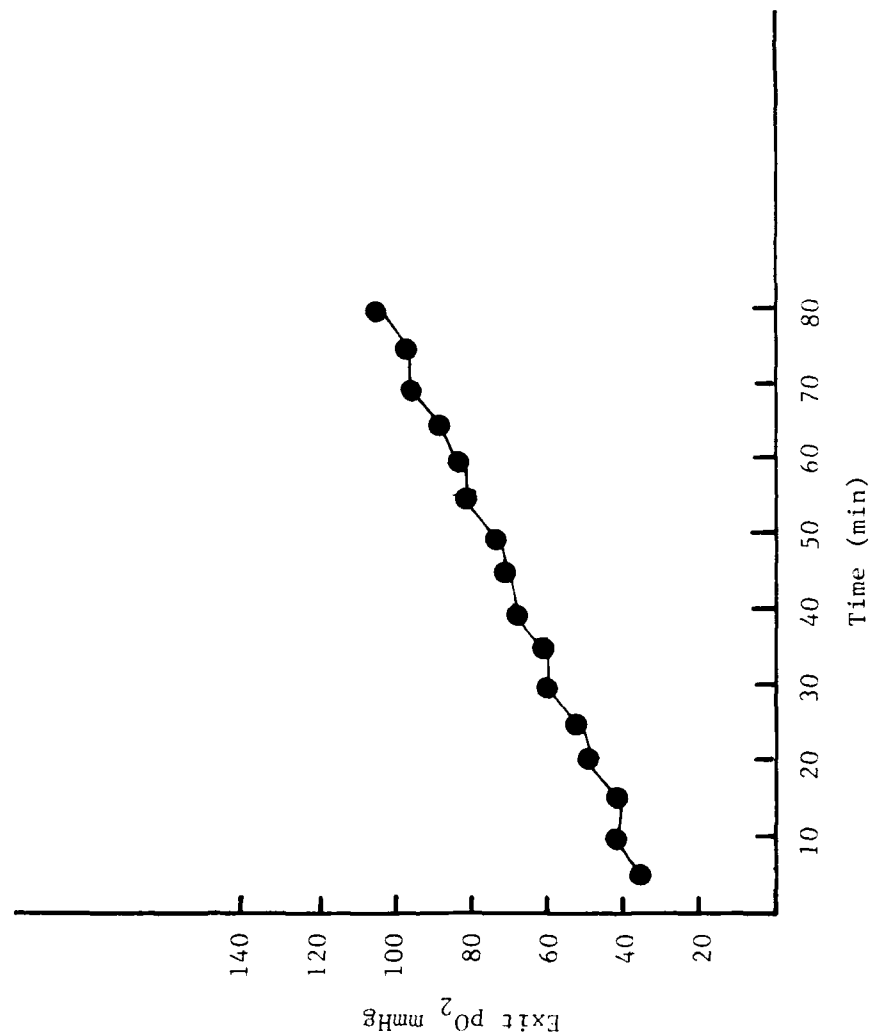


Figure 13. Change in the oxygen utilization of BHK cells after exposure to 0.65 ppm sodium cyanide: Perfusion through the bore.

BHK cells on a GSRI #1 culture unit were exposed to DMEM, 1% CS, 20 mM Hepes containing 10^{-5} M sodium cyanide at time=0. The unit was perfused through the fiber bore (as in Fig. 4a) at 0.25 ml/min by use of a Harvard syringe pump.

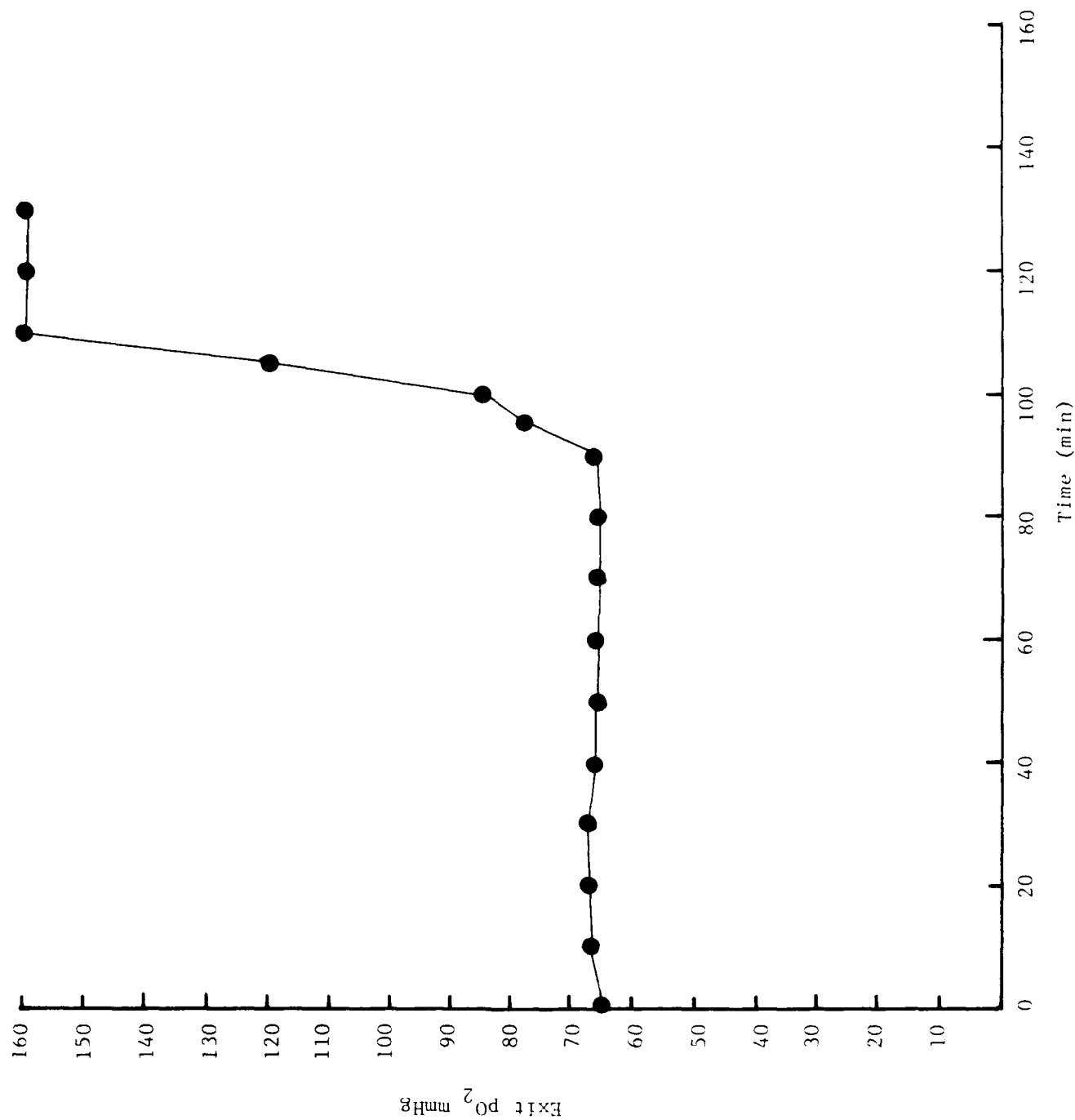


Fig. 14. Change in the oxygen utilization rate of BHK cells after exposure to 0.65 ppm sodium cyanide: perfusion through the shell.

Fig. 14. Change in the oxygen utilization rate of BHK cells after exposure to 0.65ppm sodium cyanide: perfusion through the shell.

BHK cells on a GSRI #1 culture unit were exposed to cyanide-containing medium at $t=90$ min by perfusion through the unit shell (perfusion mode shown in Fig. 4b). Perfusion was maintained at 0.25 ml/min by a Harvard syringe pump.

when exposure is immediate (Fig. 14). The result is expected since the equilibration of the bore fluid with the shell fluid is necessary in the perfusion mode of Figure 4a. In addition, the toxin concentration could be lower due to adsorption of the toxin to the capillary walls. A similar result is found for the toxic effect elicited from BHK cells by iodoacetate. Figure 15 shows the effect on oxygen utilization when the medium containing iodoacetate is perfused through the fibers only; Figure 16, the effect when the medium in the shell is replaced by iodoacetate.

The lack of response of the cells to *p*-toluidine when the medium is perfused only through the fiber bore may be due to adsorption of the *p*-toluidine by the polysulfone fibers of the Amicon unit used in these studies. Significant adsorption could prevent toxin from contacting the cells or it could lower the exposure dosage to a nontoxic level. For detection of such weak toxins, direct exposure of the cells in the capillary units may be necessary.

The reversibility of the response has been demonstrated in one experiment performed using *p*-toluidine as the toxic component in the medium (Fig. 17). In this experiment the shell medium was replaced with *p*-toluidine-containing medium (at 28 ppm) and the response was followed until oxygen utilization was almost completely inhibited. The shell medium was then replaced with medium without *p*-toluidine. A return to the preexposure utilization rate occurred.

3. CONCLUSIONS

Cultures of BHK or L929 cells can be established on the capillary culture units in one week (or less) after the initial seeding. After a plateau in growth is reached, the cells, as indicated by their metabolic rate do not seem to be harmed by a reduction in serum content of the medium from 10% to 1%. The culture can be maintained on 1% serum at a flow rate of 0.120 ml/min for at least three days using a once-through perfusion scheme. Acute toxic dosage can be sensed almost immediately by monitoring changes in the effluent medium's lactic acid or oxygen content, provided that (1) the flow rate is slow enough and (2) the toxin is in direct contact with the cells. By perfusing an established cell culture (where cells adhere firmly to the fibers) through the shell of the capillary unit, the latter condition can be met.

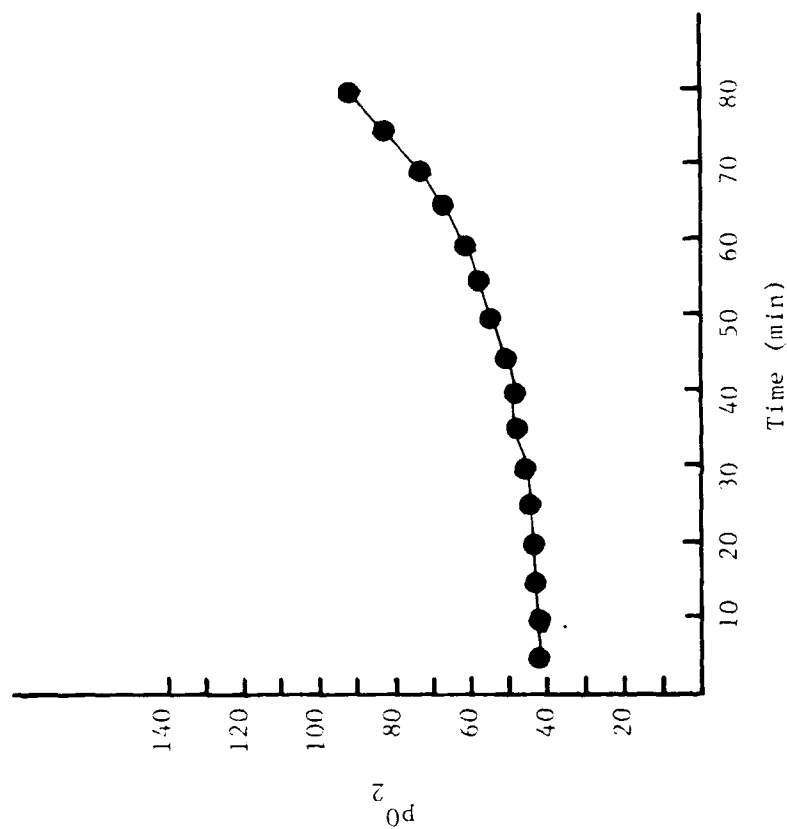


Fig. 15. Change in the oxygen utilization rate of BHK cells after exposure to 100 ppm iodoacetate; perfusion through the bore.

BHK cells on a GSRI #1 capillary unit were perfused through the fiber bore by medium containing 100 ppm iodoacetate (freshly prepared) beginning at $t=0$.

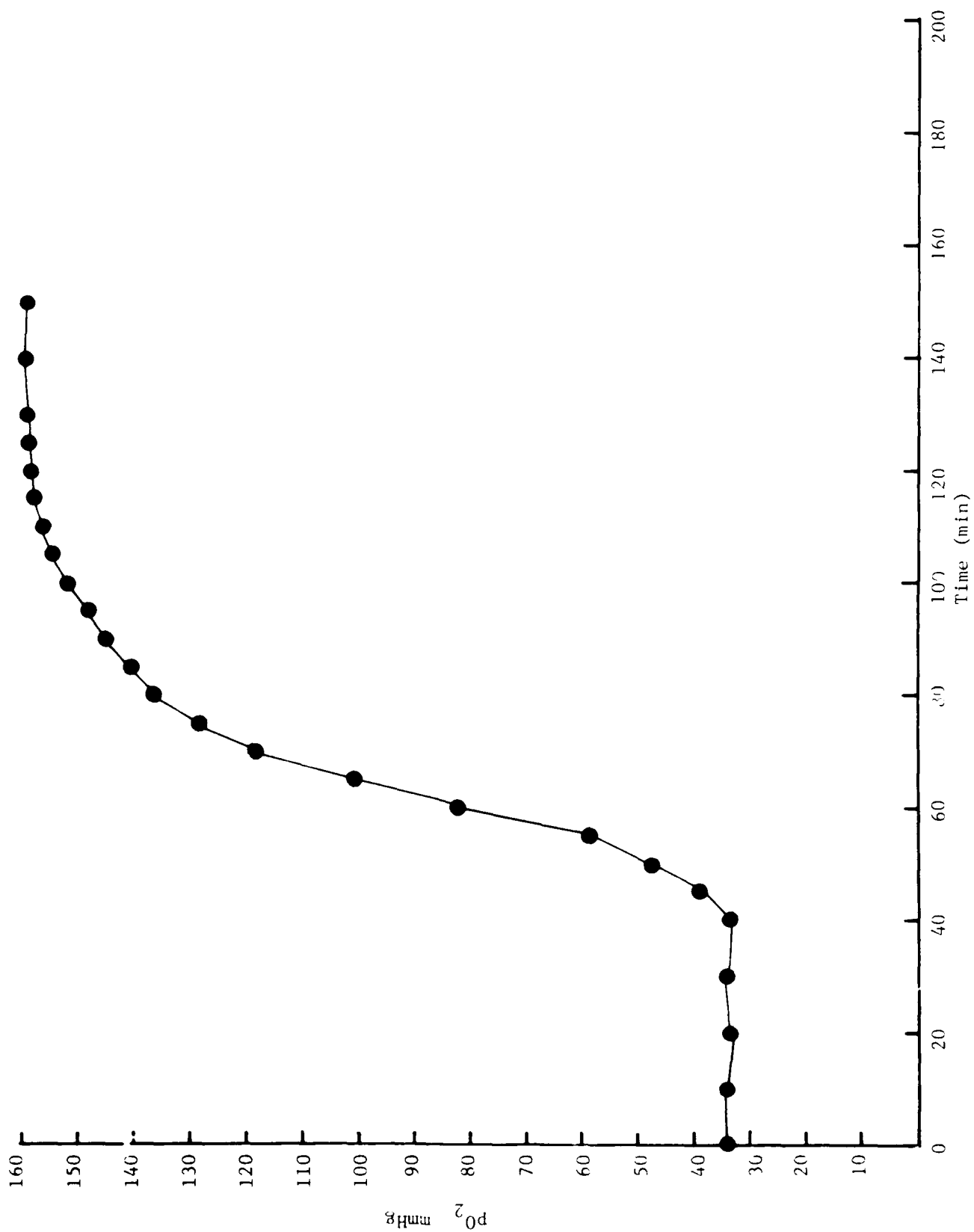


Fig. 16. Change in the oxygen utilization rate of BHK cells after exposure to 100 ppm iodoacetate; perfusion through the shell.

Fig. 16. Change in the oxygen utilization rate of BHK cells after exposure to 100 ppm iodoacetate: perfusion through the shell.

BHK cells on a GSRI #1 culture unit were perfused through the shell ports by medium containing 100 ppm iodoacetate (freshly prepared). The medium in the shell was replaced at $t-40$ min by the iodoacetate-containing medium before beginning the perfusion at a rate of 0.25 ml/min (Harvard pump).

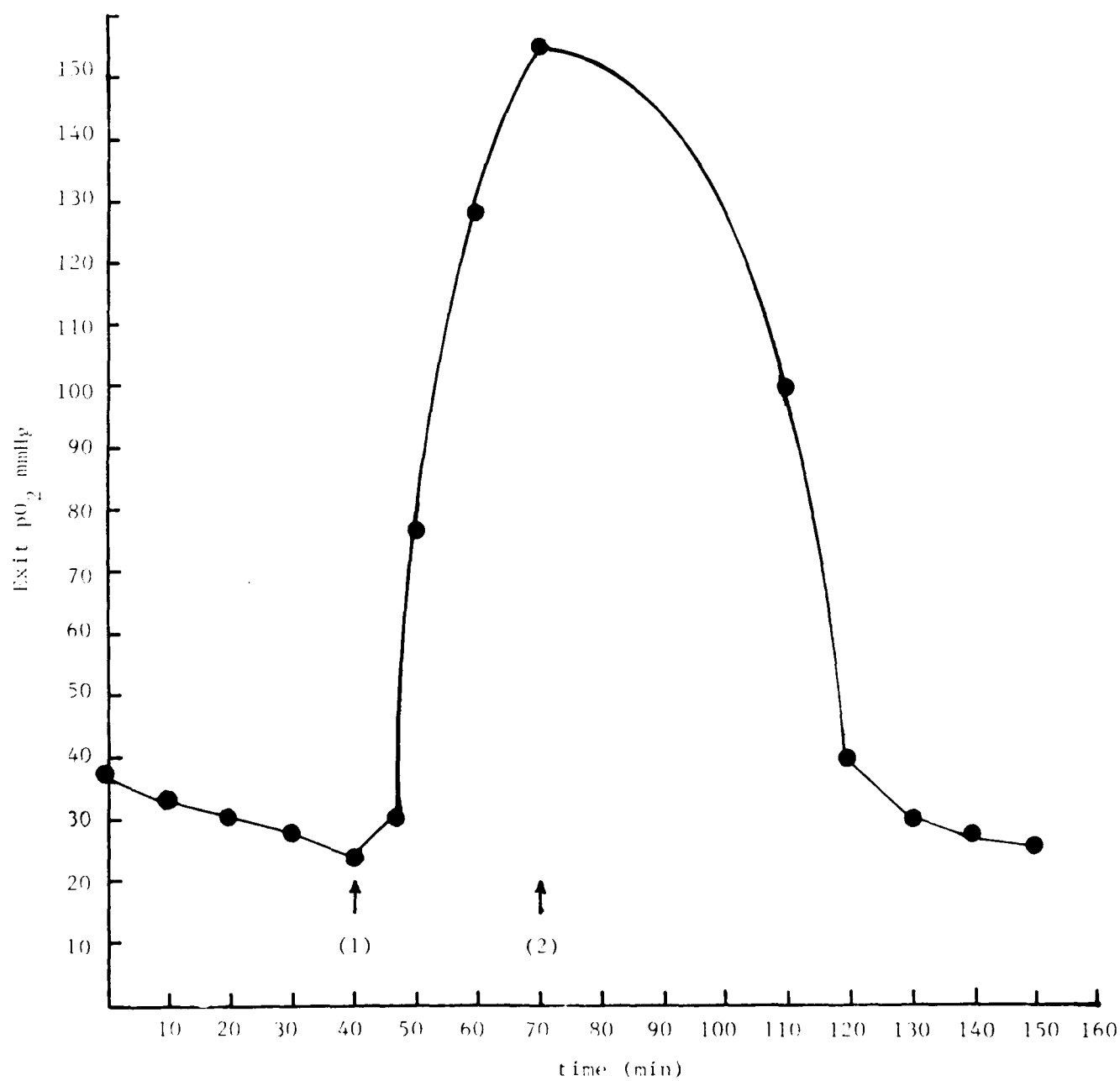


Fig. 17. Change in the oxygen utilization rate of cells after exposure to o-toluidine: Reversibility of the responses.

Fig. 17. Change in the oxygen utilization rate of cells after exposure to o-toluidine: reversibility of the response.

BHK cells on an Amicon 3S100 unit were perfused through the shell (perfusion mode of Fig. 4b) with DMEM, 1% CS, 10 mM Hepes. At the arrow #1 the medium in the shell was changed to the same medium containing 28 ppm o-toluidine and perfusion with this medium through the shell also was begun. At arrow #2 (70 min), the o-toluidine-containing medium was replaced by medium without toxin and perfusion was continued. Perfusion was at 0.27 ml/min maintained by a Sage Instruments Syringe pump.

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